Gut, 1992, **33**, 890–896

Mycobacterium paratuberculosis DNA in Crohn's disease tissue

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Abstract

Crohn's disease has long been suspected of having a mycobacterial cause. *Mycobacterium paratuberculosis* is a known cause of chronic enteritis in animals, including primates, but may be very difficult to detect by culture. IS900 is a multicopy genomic DNA insertion element highly specific for *M paratuberculosis*. A polymerase chain reaction (PCR) based on the 5′ region of IS900 and capable of the specific detection of a single *M paratuberculosis* genome was developed. This was applied to DNA extracts of full thickness samples of intestine removed at surgery from 40 patients with Crohn's disease, 23 patients with ulcerative colitis, and 40 control patients without inflammatory bowel disease. Stringent precautions were taken that excluded contamination artefact. *M paratuberculosis* was identified in 26 of 40 (65%) Crohn's disease, in 1 of 23 (4·3%) ulcerative colitis, and in 5 of 40 (12·5%) control tissues. Positive samples from Crohn's disease were from both the small intestine and colon, those from control tissues were from the colon only. All PCR internal control reactions were negative. The presence of *M paratuberculosis* in a small proportion of apparently normal colonic samples is consistent with a previously unsuspected alimentary prevalence in humans. The presence in two thirds of Crohn's disease tissues but in less than 5% of ulcerative colitis tissues is consistent with an aetiological role for *M paratuberculosis* in Crohn's disease.

Crohn's disease has long been suspected of having a mycobacterial cause.[1 2] *Mycobacterium paratuberculosis* is a known specific cause of chronic enteritis in farm and other animals,[3 4] including primates.[5] Although this organism has been cultured from a very few patients with Crohn's disease,[6–10] its role in this condition has been difficult to define. This is because of its very slow and uncertain growth in vitro culture, even from some infected animals,[11 12] and the difficulties of distinguishing it by conventional means from organisms of the *M avium* group, which are widespread in the environment[13] and normal inhabitants of the animal and human intestine.[14]

Characteristics of the subjects in each group

	Crohn's disease	*Ulcerative colitis*	*Controls*
Total no	40	23	40
Age (mean (range)) (years)	38·1 (16–79)	45·8 (14–72)	67·7 (35–92)
Male/female ratio	17/23	10/13	16/24
Small bowel	29	0	15
Colon	11	23	25

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Accepted for publication 18 February 1992

IS900 is a multicopy DNA insertion element in *M paratuberculosis* and is specific for this organism.[7 15 16] Polymerase chain reaction (PCR) amplification of IS900 provides a highly specific and sensitive method for detecting *M paratuberculosis* independent of in vitro culture.[17 18] Here we describe the application of these methods to DNA extracts of surgically resected human intestine.

Methods

PATIENTS AND SAMPLES

Full thickness samples of intestinal tissue, approximately 2 cm,[2] were obtained in the operating room immediately after surgical resection from 40 patients with Crohn's disease, 23 patients with ulcerative colitis, and 40 control patients without inflammatory bowel disease (colon cancer (35), gastric ulcer (2), sigmoid diverticulitis (2), and small gut sarcoma (1)). Tissue samples were immediately placed in dry sterile 20 ml containers and were capped, labelled, and stored in a sealed plastic bag at −20°C until processed. The diagnoses of Crohn's disease and ulcerative colitis were made on the basis of established clinical, radiological, and histological criteria. The age, sex, and site of resected tissue for each group are shown in the Table. Only obviously diseased intestine was taken in the case of Crohn's disease or ulcerative colitis. Normal intestinal tissue was sampled from control patients except for the two patients with diverticulitis in whom the sample was taken from the inflamed sigmoid colon.

ANIMAL SAMPLES

Intestinal tissue from animals with Johne's disease (ruminant paratuberculosis), confirmed by culture and histology, was kindly provided by Dr W Donachie, Moredun Research Institute, Edinburgh, UK.

TISSUE PROCESSING

Tissue was thawed, washed three times in TEN buffer (50 μM Tris/HCl, 100 mM EDTA, 150 mM NaCl, pH 8·0) and blotted repeatedly on clean filter paper paying particular attention to the mucosal surface to minimise inclusion of luminal contents. A weighed sample (1–2 g) was then chopped (McIlwain Tissue Chopper, McIlwain Laboratory Engineering, Guildford, UK) in 0·6 mm slices and homogenised (Ultra-Turrax T25) for one minute in TEN buffer (5 ml/1 g of tissue). Homogenates were divided into 500 μl aliquots for DNA extraction.

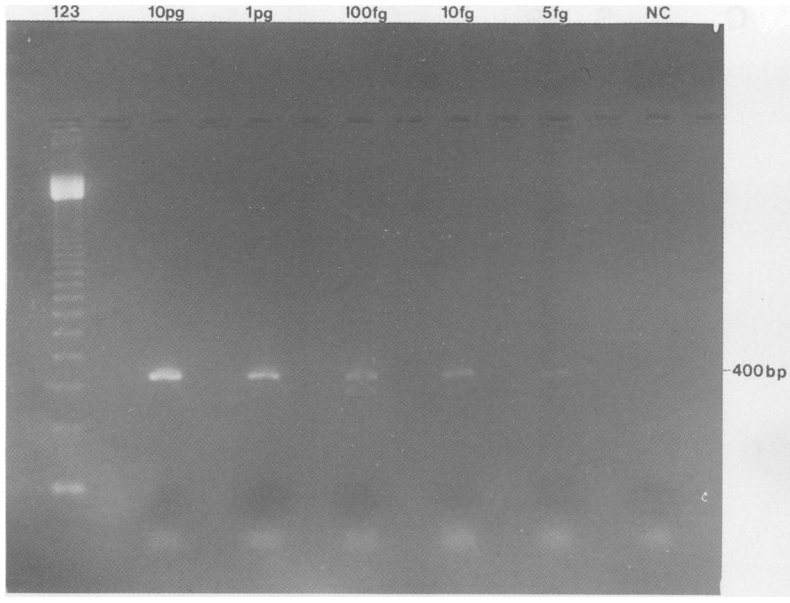

Figure 1: Detection of M paratuberculosis *DNA by IS900 polymerase chain reaction (PCR). One μl aliquots of serial dilutions of genomic* M paratuberculosis *DNA extracted from a Johne's disease derived culture were submitted to 40 cycles PCR. Ten μl of each product were run on a 2% agarose gel and stained with ethidium bromide 0·5 μg/ml 123 bp ladder (Gibco-BRL). A 400 base pair (bp) amplification product is seen in each of the sample tracks. The PCR negative control (NC) containing no template has no amplification product. Five fg of* M paratuberculosis *DNA have been detected, equivalent to a single genome in the template material added to the PCR reaction mixture.*

DNA EXTRACTION

Fifty μl of chilled lysis buffer (1% Triton X100, 10 mM EDTA, 3 mM dithiothreitol, 10 mM Tris/HCl pH 7·5) were added to a 500 μl aliquot of DNA extract, pipetted to disrupt the tissue, and centrifuged at 1500 *g* for five minutes to pellet large debris and a proportion of the eukaryotic nuclei. This step served to decrease proportionately the amount of human DNA in the final extract with a concomitant increase in sensitivity (data not shown). The supernatant was removed and submitted to DNA extraction with Subtilisin 10 mg/ml (Sigma) at 37°C for 12 hours, Lysozyme 50 mg/ml (Sigma) at 50°C for six hours, and Pronase 3 mg/ml (Sigma) 1% SDS at 37°C for 48 hours. After centrifugation at 12 000 *g* for one minute to pellet cell debris, the supernatant was subjected to two cycles of phenol/chloroform extraction (equal volumes of phenol and chloroform/isoamyl alcohol mixture 24:1 saturated with TEN) and a single chloroform extraction. The DNA was then recovered by precipitation with 2 M ammonium acetate and 2 volumes of 100% ethanol at 0°C for one hour, pelletted by centrifugation at 12 000 *g* for 20 minutes, washed in 70% ethanol, bench dried, and resuspended in 500 μl TEN buffer (10 mM Tris/HCl, 0·1 mM EDTA pH 8·8). A control buffer only tube was included with each run of homogenisation/DNA extraction.

PCR

Oligonucleotide PCR primers were selected to amplify a 400 base pair fragment (pcr400) of the 5′ portion of IS900 (nucleotides 22-421). The sequences of the primers were 5′–GTTCGGGGCCGTCGCTTAGG–3′ (Primer 90) and 5′–GAGGTCGATCGCCCACGTGA–3′ (Primer 91), (synthesised by Oswell DNA service, Department of Chemistry, University of Edinburgh, UK). Reaction mixtures were made up containing the following reagents: PCR buffer (67 mM Tris/HCl, 16·6 mM $(NH_4)_2SO_4$, 3·3 mM $MgCl_2$, bovine serum albumin (Gibco/BRL, UK) 1·7 mg/ml in TE pH 8·8), 10 mM ß- mercaptoethanol deoxynucleotide triphosphates (Pharmacia UK) 200 μM, primers 6 ng/μl, Taq DNA polymerase (Amplitaq, Cetus) 5 units, and TE buffer (10 mM Tris/HCl, 0·1 mM EDTA, pH 8·8). Five μl of the final DNA extract were added to each PCR reaction premix totalling 50 μl. A total of 33 cycles PCR were performed (Techne Thermal Cycler, PHC-1, Cambridge UK) com-

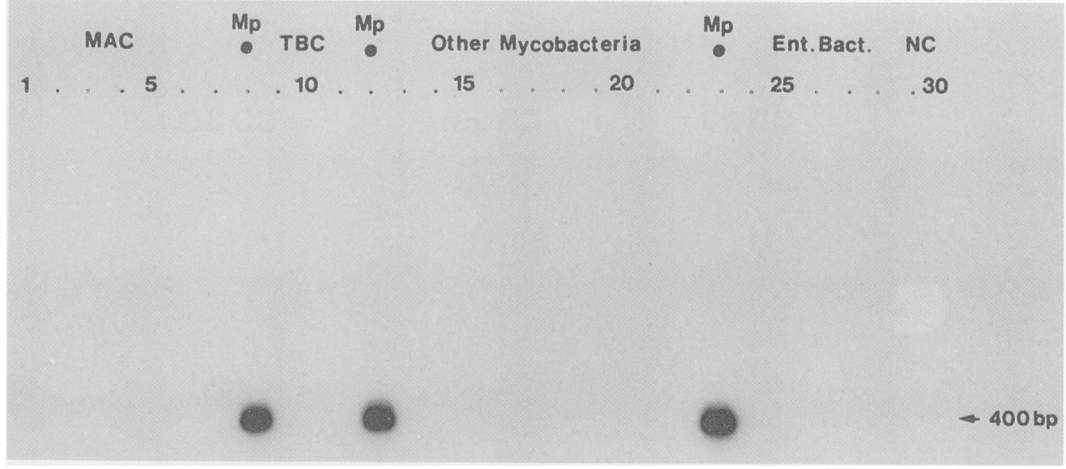

Figure 2: Autoradiograph showing IS900 polymerase chain reaction (PCR) applied to DNA extracted from 27 cultures of mycobacterial species and other bacteria. Amplification products run on 2% agarose gel after 33 cycles PCR and Southern blotted onto a nylon membrane; hybridised with ^{32}*P-dCTP labelled pcr400 product, washed at high stringency, and exposed for four hours. MAC –* M avium *complex, TBC –* M tuberculosis *complex. A positive signal is seen only in the three* M paratuberculosis *(Mp) lanes 8, 12, and 23. Other lanes are as follows: 1 and 2 –* M avium*; 3 to 5 –* M avium *AIDS isolates servovars 9,1 and 6; 6 –* M scrofulaceum*; 7 –* M avium *subsp silvaticum (woodpigeon)*[20-22] *containing related insertion element IS902*[23]*; 9 –* M tuberculosis*; 10 –* M africanum*; 11 –* M bovis*; 13 –* M Kansasii*; 14 –* M fortuitum*; 15 –* M xenopi*; 16 –* M gordonae*; 17 –* M malmoense*; 18 –* M phlei*; 19 –* M marinum*; 20 –* M chelonei*; 21 –* M smegmatis*; 22 –* M szulgai*; 24 – Anaerobic peptococcus; 25 –* Streptococcus faecalis*; 26 –* Escherichia coli*; 27 –* Streptomyces coelicolor *containing related insertion element IS110*[24]*; 28 – buffer only DNA extraction control; 29 and 30 – PCR negative control (NC).*

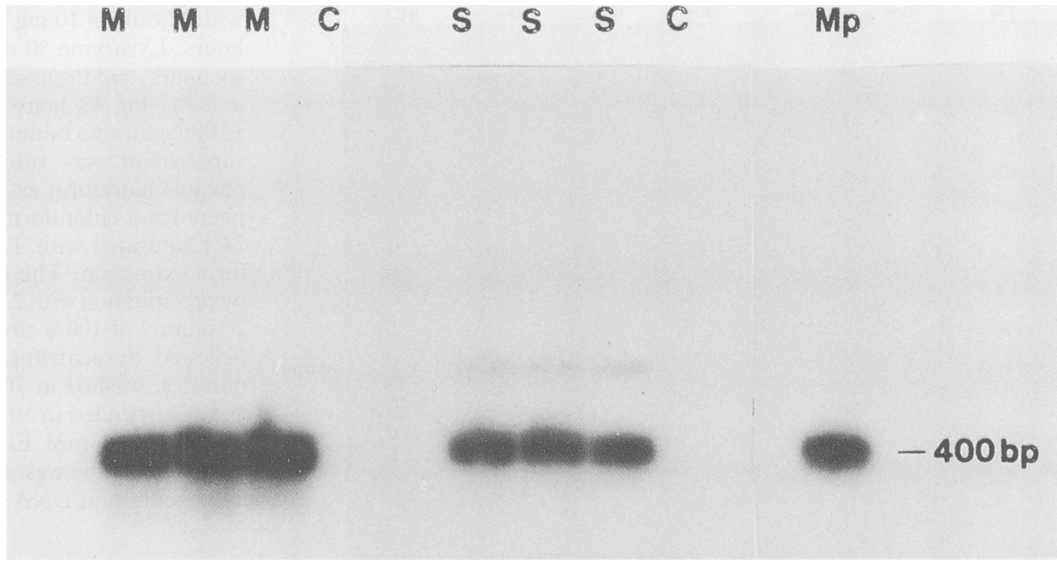

Figure 3: Autoradiograph showing detection of M paratuberculosis *DNA in tissue DNA extracts of intestinal tissue from a goat (M) and a sheep (S) with Johne's disease. Five μl of a 10^5 dilution of M and 5 μl of a 10^7 dilution of S were submitted to 33 cycles IS900 polymerase chain reaction (PCR) amplification, in triplicate, and each with one buffer only negative control (C). Ten fg of* M paratuberculosis *DNA (Mp) were amplified as a positive control. PCR products were run on a 2% agarose gel, Southern blotted onto a nylon membrane, and hybridised at high stringency with ^{32}P labelled pcr400 product. The membrane was exposed to Kodak X-omat film for one hour*

prising denaturation at 95°C for one minute, annealing at 58°C for one minute, and extension at 72°C for three minutes. Each tissue sample was run in triplicate with one buffer only PCR negative control. Each experiment included one positive control tube containing 10 fg known *M paratuberculosis* DNA.

CONTAMINATION PRECAUTIONS

Stringent precautions were taken throughout all tissue processing, DNA extraction and PCR steps to avoid false-positives because of laboratory contamination. These precautions included the use of widely separated contained laboratories for individual procedures, the use of dedicated or disposable equipment, ultraviolet irradiation of buffers and surfaces, capped PCR pre-mix tubes opened on a single occasion only for 5 μl sample addition and the meticulous cleaning of surfaces, pipettes, and other apparatus with 1 M HCl, 10% hypochlorite, or 100%

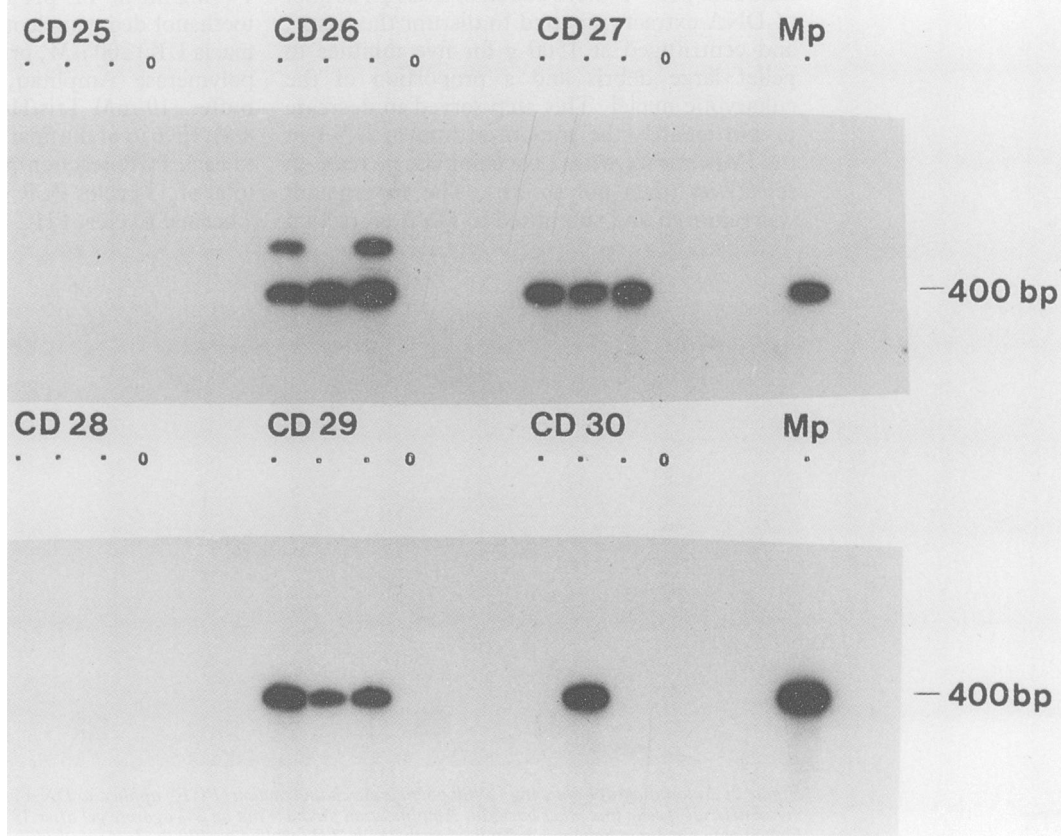

Figure 4: Autoradiographs showing detection of M paratuberculosis *DNA in four of the six Crohn's disease tissue DNA extracts shown here. Samples were submitted to 33 cycles polymerase chain reaction (PCR) amplification in triplicate, each with one buffer only PCR negative control sample (o). Ten fg of* M paratuberculosis *DNA were amplified as a positive control (Mp). PCR products were run on 2% agarose gel, Southern blotted onto a nylon membrane, and hybridised at high stringency with ^{32}P labelled pcr400 product. The membrane was exposed for 12 hours on Kodak X-omat film. CD26, CD27, and CD29 show triplicate positive signals. CD30 shows a single positive signal suggesting a lower abundance of* M paratuberculosis *genomes. The additional higher molecular weight band in CD26 is seen with some* M paratuberculosis *isolates and may be PCR over-amplification.*

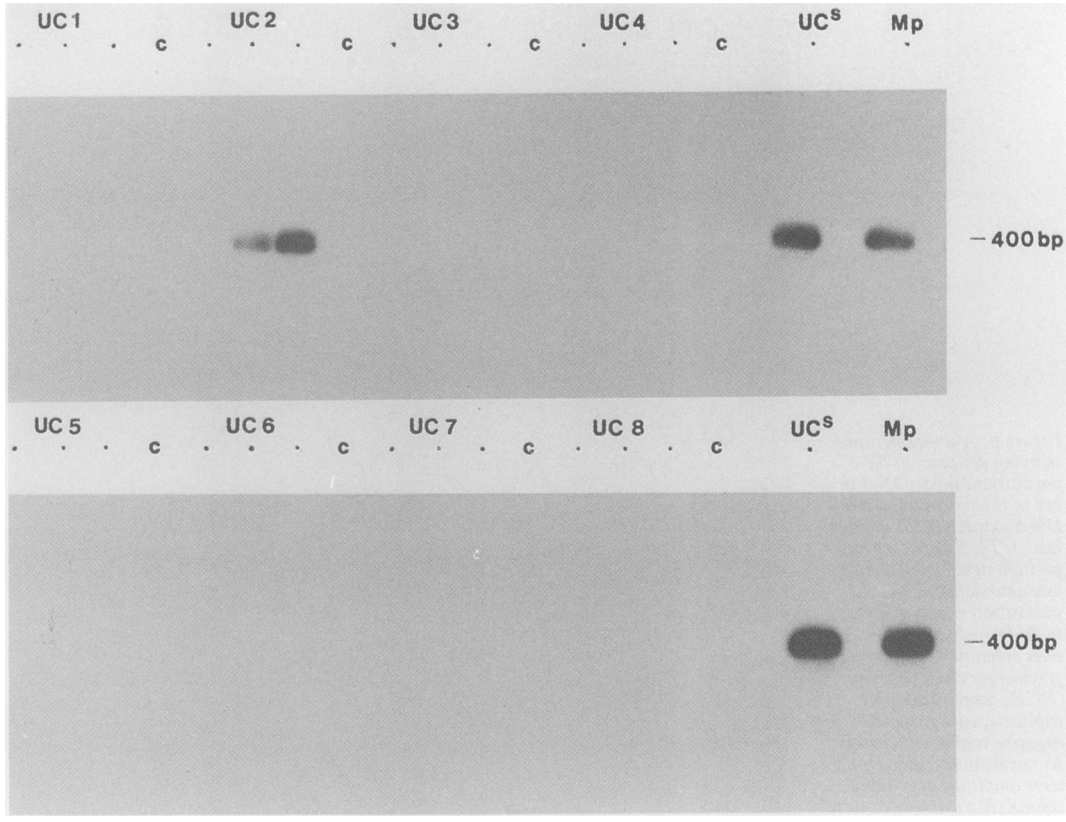

Figure 5: Autoradiographs showing detection of M paratuberculosis *DNA in one of the eight ulcerative colitis tissue DNA extracts shown here. Samples were submitted to 33 cycles polymerase chain reaction (PCR) amplification in triplicate, each with one negative control (c). UC2 shows positive signals in two out of three sample lanes. All other samples are negative. Ten fg* M paratuberculosis *DNA were amplified as positive control (Mp). Two ulcerative colitis samples (UC^S) have been spiked with 10 fg* M paratuberculosis *DNA to exclude inhibition in the assay. Both show positive signals equivalent to the 10 fg* M paratuberculosis *DNA alone.*

ethanol. These measures were monitored by the inclusion of TEN buffer blanks subjected to all steps in the tissue preparation and DNA extraction and the inclusion of a no template PCR control for each tissue sample.

SOUTHERN BLOT HYBRIDISATION

PCR products were run on 2% agarose gels (Sigma/Nu-sieve) and Southern blotted onto nylon membranes (Hybond-N, Amersham UK). Membranes were hybridised at 65°C overnight with the 400 base pair PCR product or a 228 bp probe from within this fragment, labelled with ^{32}P dCTP (Multiprime DNA labelling kit, Amersham UK), in a buffer containing 3xSSC, 0·1% bovine serum albumin (fraction V, Sigma), 0·1% Ficoll (Pharmacia UK), 0·1% polyvinylpyrrolidone (Sigma), 0·5% sodium dodecyl sulphate (SDS) (Sigma), 10% dextran sulphate (Pharmacia UK), and 100 μg/ml sheared and denatured salmon sperm DNA (Sigma). The membranes were then washed at 65°C in 3×SSC 0·1% SDS for 3×30 minutes, 1×SSC 0·1% SDS for 30 minutes, and 0·1×SSC and 0·1% SDS for 30 minutes, and exposed on X-Omat film (Kodak) at −70°C for 12 and 60 hours.

A positive result in the IS900 PCR test was indicated by the presence of the correct 400 bp band in any one of the triplicate test lanes, or in any control lane.

SENSITIVITY OF TISSUE DETECTION

Five μl (1%) of the final tissue DNA extract were used in each of the triplicate PCR reaction tubes, which together were equivalent to about 0·003 g of original tissue. Given the known PCR sensitivity (and assuming 100% efficiency in the DNA extraction), the overall limit of detection was estimated to be about 300 bacilli per 1 g of tissue.

STANDARD M PARATUBERCULOSIS DNA

A goat Johne's disease derived *M paratuberculosis* strain was kindly supplied by Dr Martin Woodward, Central Veterinary Laboratories, Weybridge, Surrey, UK. This was cultured on a mycobactin enriched Lowenstein-Jensen slope. The DNA was extracted enzymatically as above and the final concentration assessed by comparison with standard DNA solutions of known concentration on an ethidium bromide stained agarose gel and by optical density analysis at OD260.

MYCOBACTERIAL DNA SAMPLES

Broth and solid media cultures of different mycobacteria, enteric bacteria, and streptomyces (see legend to Fig 2) were used to demonstrate specificity of the IS900 PCR. Cell pellets from in vitro cultures were subjected to boiling for 20 minutes to release the microbial DNA, and 5 μl aliquots of the resultant solution were used in PCR reactions. The availability of each bacterial DNA for the PCR IS900 test was confirmed by subsequent PCR using primers amplifying a conserved region of the 16S rRNA gene[19] (data not shown).

Results

The IS900 PCR assay detected 5 fg of the stock

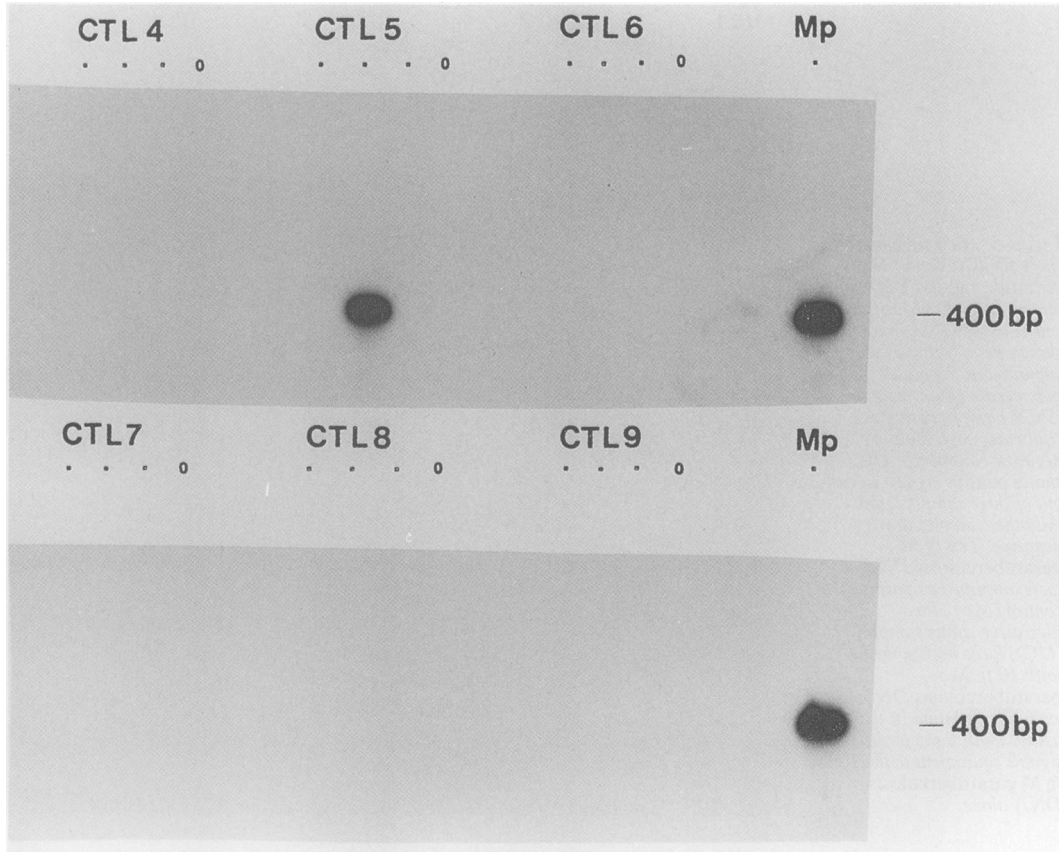

Figure 6: Autoradiographs showing detection of M paratuberculosis *DNA in one of the six control tissue DNA extracts (CTL) shown here. CTL 5 shows a single positive signal suggesting a low abundance of* M paratuberculosis *genomes in this sample. Samples were submitted to 33 cycles polymerase chain reaction (PCR) amplification in triplicate, each with one negative control (o). Ten fg* M paratuberculosis *DNA were amplified as positive control (Mp). Products were Southern blotted and hybridised as for Figure 3.*

M paratuberculosis DNA, equivalent to a single mycobacterial genome. After 40 cycles PCR this result could be visualised on an agarose gel stained with ethidium bromide (Fig 1).

As expected, all the known *M paratuberculosis* isolates were IS900 PCR positive. IS900 PCR did not amplify a 400 bp product or show a hybridisation signal with any of the 24 other mycobacterial or enteric bacterial DNA extracts (Fig 2). The availability of bacterial DNA in each of these extracts was confirmed by a strongly positive PCR reaction using the 16S rRNA primers.[19]

The correct 400 bp amplification product was obtained in each case when the IS900 PCR was applied to the DNA extracted from goat and sheep Johne's disease intestinal tissues, following the procedure identical to that used for the human tissues (Fig 3). The native tissue DNA extracts had, however, to be diluted by a factor of 10^5 for goat and 10^7 for the sheep, to reduce the intensity of the IS900 PCR signal to approximate that obtained with the positive internal control reactions in the experiments.

Twenty six of the 40 (65%) Crohn's disease intestinal tissues were positive for *M paratuberculosis* (Fig 4); of these 19 were from the small intestine and seven from the colon. One of 23 (4·3%) ulcerative colitis tissues (Fig 5) and 5 of the 40 (12·5%) control tissues were also positive (Fig 6). The five control samples that were positive were all from histologically normal colon in patients with colon cancer. Thirteen of the 26 positive Crohn's disease tissue samples were positive in two or all three lanes in the triplicate assay; the five positive control tissues were positive in one lane only. Both diverticulitis samples were negative. All of the 103 buffer only PCR control tubes and the 11 homogenisation/DNA extraction blanks were negative. All of the known *M paratuberculosis* positive internal controls, were positive.

In five of the positive Crohn's disease patients selected at random, we returned to the original stored tissue. The DNA extraction and IS900 PCR were repeated with simultaneous internal PCR controls as before. All the Crohn's disease samples were again positive and the PCR controls negative. Four PCR negative Crohn's disease, two ulcerative colitis, and two control tissue DNA extracts were spiked with 10 fg *M paratuberculosis* DNA (equivalent to 2 mycobacterial genomes) and submitted to PCR to exclude inhibition in the assay. A positive signal was obtained in each case equivalent to that from 10 fg *M paratuberculosis* DNA alone. PCR positive or negative results in the 40 Crohn's disease patients showed no relation with small or large gut involvement or the presence or absence of granulomata.

Discussion

The specificity for *M paratuberculosis* of PCR assays based on their IS900 DNA insertion elements has been reported from work done in our own and other laboratories.[17,18] This is again demonstrated in the present study using the oligonucleotide primers and PCR assay conditions described. In addition, we show that the IS900 PCR reaction does not report the very closely related *M avium* subsp silvaticum.[20] This is the 'woodpigeon' strain of *M avium*, also known to be a specific cause of chronic enteritis

in cattle.[21 22] *M avium* subsp silvaticum contains 10 or 11 copies of IS902,[23] a second member of this unusual family of mycobacterial DNA insertion elements with close structural homology to IS900 in *M paratuberculosis*.

Oligonucleotide primers previously described for the PCR detection of *M paratuberculosis*[17] were found not to be optimal for the human tissue DNA extracts. In the present study primer sequences were selected on the basis of minimal 3′ base mismatch, freedom from involvement in IS900 secondary structure, and specificity in relation to the known DNA sequences of other elements in this family.[15 23-26] Reaction conditions were optimised and maintained throughout the study, such that the IS900 PCR assay comfortably reported 10 fg of target *M paratuberculosis* DNA or less. This sensitivity, together with meticulous DNA extraction methods and other conditions described, were found to be important since if assay performance and sensitivity drifted, reproducible results were no longer assured. Insufficient overall sensitivity in the detection of *M paratuberculosis* in a small number of fresh or fixed tissues probably accounts for the inability of some preliminary studies to detect this agent in Crohn's disease intestine[27 28]; another study was positive.[29]

Throughout the present investigation, the results of IS900 PCR were clear cut. Either we saw a blank lane or the distinct 400 bp band hybridising specifically with the specific ^{32}P-PCR probe at high stringency (Figs 4 to 6). The number of positive lanes in each triplicate is a reflection of tissue microbial abundance. The extensive precautions adopted and the uniformly negative internal PCR control reactions strongly suggest that the positive results obtained in the PCR assays were not artefacts of laboratory contamination (well known in PCR systems,[30 31]) but correctly indicated the presence of IS900 containing *M paratuberculosis* DNA in the corresponding tissue extracts.

The true distribution of the animal enteric pathogen *M paratuberculosis* in the environment, in water supplies, and in human foods[32] is largely unknown because of the need hitherto to rely on the uncertainties of isolation and conventional characterisation in in vitro culture.[33 34] The finding of the DNA of this organism in a small proportion of samples of apparently normal colonic tissue including mucosa, is consistent with a previously unsuspected alimentary prevalence in humans. If the presence of *M paratuberculosis* in the inflamed intestinal tissues of most patients with Crohn's disease in the present study was a consequence of opportunistic invasion due to pre-existing disease, we could reasonably expect to find a broadly similar situation in intestine affected by ulcerative colitis (or diverticulitis). The low proportion of positive results in this group (4·3%) favours an aetiological association between *M paratuberculosis* and the chronic enteritis of Crohn's disease in humans.

Although far short of proof, our findings are consistent with the possibility that a majority of Crohn's disease in southern England may be caused by *M paratuberculosis*, in a tissue abundance one hundred thousand to 10 million times lower than that commonly occurring in correspondingly diseased animals. A tissue microbial abundance below the limit of detection by our present methods may account for some of the PCR negative results on Crohn's disease patients. An additional and equally probable explanation would be the involvement of one or two other members of a small group of environmental mycobacteria very closely related to *M paratuberculosis*, and also specifically capable of causing chronic enteritis in an appropriately susceptible host. This would be entirely compatible with the known clinicopathological heterogeneity of Crohn's disease. Methods similar to those used in the present study will need to be applied to investigate these agents.

The immunological detection of *M paratuberculosis* in humans has so far been inconsistent or negative.[35-37] This may reflect the phenotype of this organism in the human host and the difficulties of differentiating specific responses to *M paratuberculosis* from existing immunity to *M avium* and other mycobacteria widespread in the environment and in ourselves. Epitope mapping and the availability of chemically defined sequences within the p43 protein encoded by IS900 may offer a promising way forward in this field.

This work was supported by grants from the Ileostomy Association of Great Britain and Ireland, Action Research, and the Wellcome Trust, to whom we express our appreciation. We are grateful to many colleagues for contributing tissue samples.

This work was previously outlined in abstract form. Sanderson *et al*. Polymerase Chain Reaction (PCR) directly reports Mycobacterium paratuberculosis (M para) genomes in Crohn's disease tissue DNA extracts. *Gastroenterology* 1991; **100**: A247.

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