Specific detection of *Mycobacterium paratuberculosis* DNA associated with granulomatous tissue in Crohn’s disease

H M Fidler, W Thurrell, N Mcl Johnson, G A W Rook, J J McFadden

Abstract
The role of mycobacteria, specifically *Mycobacterium paratuberculosis*, in Crohn’s disease has aroused considerable controversy for many years. Using the ultra sensitive polymerase chain reaction some studies have reported detection of *M paratuberculosis* DNA in as many as 65% of Crohn’s disease patients but also in patients without disease. Other studies have been negative for both groups. We therefore designed a double blind control trial to investigate the presence of mycobacterial DNA in age, sex, and tissue matched paraffin wax embedded tissues from 31 Crohn’s disease tissues, 20 diseased gut control tissues, and 10 ulcerative colitis tissues. The specimens were coded and analysed blind with three separate polymerase chain reactions (PCR) based on DNA sequences specific for *M paratuberculosis* (IS900), *M avium* (RFLP type A1) (IS901), and the *Mycobacterium* genus (65 kDa gene, TB600). The number of granulomata and presence of acid fast bacilli in each Crohn’s disease tissue was also investigated. The sensitivity of the system was determined using similarly prepared gut tissue from an animal infected with *M paratuberculosis*. Four of 31 Crohn’s disease tissues and none of the 20 control and ulcerative colitis derived tissues amplified *M paratuberculosis* DNA. Crohn’s disease tissues containing granulomata were significantly more likely to amplify *M paratuberculosis* specific DNA on PCR than the non-Crohn’s disease tissues (p=0.02). All the positive Crohn’s disease tissues contained granulomata, and none contained acid fast bacilli. Equivalent numbers of Crohn’s and non-Crohn’s disease tissues amplified the region of the 65 kDa gene on PCR for non-specific mycobacterial DNA (11/31 and 9/30 respectively). No sections produced an amplified product with the IS901 PCR. These results suggest that few Crohn’s disease gut biopsy sections contain *M paratuberculosis* DNA in association with granulomata. The absence of such DNA in any control and ulcerative colitic tissue strengthens the case for it having a specific association, which may be pathogenic, with Crohn’s disease in this minority of patients.

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Crohn’s disease is a distressing disorder with a significant morbidity and mortality in a young population. With no convincing aetiology established, a search for effective treatment is severely limited. The clinical and pathological similarities between both tuberculous ileitis in man and Johne’s disease, a chronic enteritis in ruminants caused by *Mycobacterium paratuberculosis*, aroused speculation of a mycobacterial aetiology from the first descriptions of Crohn’s disease. As no acid fast organisms could be directly detected, little progress was made until mycobacteria were cultured from the gut tissue of affected subjects. Although a number of mycobacterial species have since been cultured from Crohn’s disease tissue, the isolation of *M paratuberculosis* by four independent laboratories, has aroused the greatest interest. Recently, a second mycobacterial strain that causes Johne’s disease in animals, *M avium* (RFLP type A1), has also been cultured and identified, from Crohn’s disease tissue. When first isolated from Crohn’s disease tissue, *M paratuberculosis* seems to be in a very slow growing, spheroplast like form.

These cell-wall deficient spheroplasts have variable acid fast staining properties and would have been easily missed by conventional microbiological detection. The isolation of *M paratuberculosis* remains entirely specific to Crohn’s disease – no control specimen has yielded *M paratuberculosis* on culture. *M paratuberculosis* has also never been isolated from AIDS patients who often suffer gastrointestinal and disseminated infections as a result of the very closely related *Mycobacterium avium*. Less than 5%, however, of Crohn’s disease tissue specimens that have been cultured have yielded *M paratuberculosis*. The extremely long incubation times (sometimes greater than one year) and poor recovery of *M paratuberculosis* by culture from Crohn’s disease tissue has prompted the use of DNA probe technologies. The ultrasensitive technique of the polymerase chain reaction (PCR) has been applied directly to Crohn’s disease tissues. Using this technique, two studies have reported negative findings for the presence of *M paratuberculosis* DNA in Crohn’s disease tissue while one study reported detection of *M paratuberculosis* DNA in 65% of Crohn’s disease tissues, but significantly, in 12% of normal gut. In this second study, replicate experiments did not always give concordant results, presumably because of sampling errors caused by the very low numbers of organisms present in each aliquot of tissue extract.

Critical to an evaluation of the possible role of *M paratuberculosis* in Crohn’s disease is the specificity of its association with the disease and to the lesions characteristic of the disease. We therefore designed a double blind control study of Crohn’s disease, ulcerative colitis, and inflamed gut controls using histologically...
examine paraffin wax embedded sections as source material. We used three PCR reactions, the first specific to *M. paratuberculosis*, the second specific to *M. avium* (RFLP type A/1), and lastly, a PCR reaction designed to amplify a segment of the mycobacterial 65 kD antigen gene (TB600). We have previously shown that this 65 kD PCR detects DNA from all mycobacterial species we have examined but does not amplify non-mycobacterial DNA. We used similarly prepared *M. paratuberculosis* infected Johne’s disease tissue as a control for sensitivity (kindly donated by I Euan Jones, Veterinary Investigation Centre, Aberystwyth). Finally, we investigated, again in a blinded experiment, if the number of granuloma in the section was related to positivity by PCR, and whether PCR positive Crohn’s disease tissue was also positive by acid fast staining.

**Patients and methods**

**PATIENTS**

Samples from 61 patients who had had surgery and biopsy at the Middlesex Hospital or Whittington Hospital between 1989 and 1992 were used. These were paraffin wax embedded gut tissue from endoscopic biopsy or surgical resection specimens. Thirty one of these were from well recorded Crohn’s disease patients, 20 from control patients with inflamed gut because of ileostomies, radiation damaged gut, carcinomas, vaginoplasties or Meckel’s diverticula, and 10 from patients with ulcerative colitis (Table 1). The Crohn’s tissue and non-Crohn’s disease tissue groups were roughly matched by age, tissue, racial origin, and type of section. For all tissues the preservative method was immersion in buffered neutral formalin for up to 24 hours followed by embedding in paraffin wax.

**PREPARATION OF SAMPLE DNA**

Sections of 25 μm were cut from each block with a sterile ‘no touch’ technique, and new blades were used for each to reduce contamination. These sections were then placed in sterile 1.5 ml Eppendorf containers and allocated a code number by the histopathology department at the corresponding hospital. The samples were then analysed at Surrey University, where they were deparaffinised, DNA was extracted, and was purified as previously validated in our laboratory before polymerase chain reaction techniques.

**POLYMERASE CHAIN REACTION**

Three sets of primers were used. IS900 is specific for *M. paratuberculosis* and is present in multiple copies of 15–20 genome. It has been validated for the specific detection of *M. paratuberculosis* organisms in Johne’s disease, and used in the detection of *M. paratuberculosis* in both laboratory cultures and Crohn’s tissue. Primers were designed to amplify a 354 base pair product from position 209 of the 5’ end of the 1451 base pair sequence. IS900(1): 5’ TGAGCAATGACG-GTTACGGAGGGT 3’; IS900(2): 5’ TGATGCAGCTTTGCGTGTCGGT 3’. Primers were also used from the insertion sequence IS901, which is specific for *M. avium* RFLP type A/1, which includes the wood pigeon bacillus, and has also been implicated in Crohn’s disease. These amplify a 1108 base pair product. IS901(1): 5’ TGAGCAACCGTTGT-GTCATTGAAA 3’; IS901(2): 5’ TGATAVCG-GCGGGAATCGCCGT 3’.

Finally, primers were made from a region of mycobacterial specific (65 kD gene, TB600) gro EL gene, which is conserved among known mycobacteria but not specific for any.” They produce a product with 625 base pairs. TB 1: 5’ GAGATCGAGCTGAGGATTCCGTACG 3’; TB 2: 5’ GCGGATCTTTGTTGACGACCAGG 3’.

Amplification was performed as described elsewhere, but substituting deoxythymidine triphosphate with deoxycytidine triphosphate in the deoxynucleotide mixture. This results in an amplified product, which can be degraded enzymatically before the subsequent PCR and provides an extra reduction in contamination, which complements obsessional housekeeping practices. The amplified products were electrophoresed through a 1.5% agarose gel stained with ethidium bromide and viewed under ultraviolet light. Positive controls of 6 fg, 6 fg, and 125 fg of *M. paratuberculosis*, *M. avium* (RFLP type A/1), and *M. tuberculosis* DNA respectively were run for IS900, IS901, and TB1 and two primers to reflect their differing sensitivities. For greater sensitivity we then performed Southern hybridisation and probing as previously described. This procedure was performed for all specimens and all DNA products using digoxigenin labelled probes. To detect contamination during sample preparation, negative control samples containing only sterile water were treated exactly as the tissue samples and examined by PCR in parallel. One negative control sample was processed for every six tissue samples examined, for every PCR run with all three primer pairs.

**HISTOLOGICAL EXAMINATION**

PCR positivity may be related to the degree that each tissue section is affected by disease. An independent histopathologist, unaware of the PCR results, therefore counted the number of granuloma present in an adjacent section of each Crohn’s tissue block subjected to PCR. In addition, sections from each Crohn’s tissue block were Ziehl-Neelsen stained for the presence of acid fast bacilli.

**Results**

**SENSITIVITY OF IS900 PRIMERS**

We have previously detected 6 fg (corresponding to one mycobacterial genome) of purified *M. paratuberculosis* DNA using the above system. We were concerned that our sensitivity for intact organisms in situ may be different from that for purified DNA. We therefore used similarly prepared paraffin wax embedded Johne’s tissue from sheep gut, characteristically containing many *M. paratuberculosis* organisms (kindly donated by I W Jones, Veterinary Inves-
TABLE I  Origin and patient characteristics of selected paraffin wax embedded tissues

<table>
<thead>
<tr>
<th>Tissue type</th>
<th>Total</th>
<th>Age (y) (SD)</th>
<th>Sex ratio</th>
<th>M/F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crohn’s disease</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14 small bowel</td>
<td>31</td>
<td>42 (20)</td>
<td>10M/21F</td>
<td></td>
</tr>
<tr>
<td>16 large bowel</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 anal tag</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ulcerative colitis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 small bowel</td>
<td>10</td>
<td>49 (14)</td>
<td>7M/3F</td>
<td></td>
</tr>
<tr>
<td>7 large bowel</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>13</td>
<td>45 (19)</td>
<td>8M/12F</td>
<td></td>
</tr>
<tr>
<td>6 small bowel</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 anal tag</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

gitation Centre, Aberystwyth) and applied identical DNA extraction and amplification techniques to it. By estimating the number of organisms in the original section (80 million), and serially diluting the purified extracted DNA in a mycobacterium free similar preparation of gut DNA, we could estimate the number of organisms per human gut section. We found that we could detect a one million-fold dilution on an agarose gel (Fig 1) and a 10 million-fold dilution by Southern hybridisation, of Johne’s tissue diluted in human gut DNA extract (Fig 2).

The sensitivity of the IS900 PCR for M. paratuberculosis organisms in gut tissue was therefore about 8-80 organisms/paraffin wax embedded tissue section (equivalent to about 30 million human cells assuming 100% DNA extraction). As only 1/10 of the specimen was examined in any experiment, this was a similar sensitivity to that obtained for purified DNA (about one genome) and similar to that obtained in other studies.

All water internal control samples with each PCR were always negative, suggesting that we had no contamination using our system. This is attributable to the considerable efforts we have made to avoid contamination and the use of deoxyuridine triphosphate in our reaction vessels. In a separate experiment we confirmed the absence of PCR inhibition in our samples by spiking each with M. paratuberculosis DNA and ensuring the appropriate product was amplified.

PCR RESULTS

Four of 31 Crohn’s disease tissues that received PCR amplified IS900 (Fig 2). All positive samples were confirmed by repeat testing. As each sample was eluted in 200 μl of TRIS-EDTA buffer and only 2 μl used per reaction, we estimate that at least 100 copies of the M. paratuberculosis genome were present in each of these sections. One sample (lane 15, Fig 2) continued to amplify IS900 on PCR after a further 20-fold dilution, suggesting that 2000 copies may have been present in the original section. Only two of these four positive tissues required Southern hybridisation to detect the amplified product. All four positive sections were from large bowel sections, one from a colonic endoscopic biopsy, one from a rectal endoscopic biopsy, and two from rectal resections. No sample from the control group or the ulcerative colitic group amplified IS900 after PCR.

Table II gives the results for the TB600 (65 kD gene), which detect any mycobacterial DNA present. Eleven of 31 Crohn’s disease tissues, including all four that amplified IS900 (35%), 1/10 ulcerative colitis tissues (10%), and 8/20 (40%) control tissues produced an amplified product of the appropriate size after PCR. Both small and large bowel contained this sequence for the Crohn’s disease patients and controls. No sample received amplification with the IS901 primers.

Histological examination of the 31 Crohn’s disease tissue specimens identified granuloma in 20 sections. These included 7/14 small bowel sections, 12/16 large bowel sections and 1/1 anal tags. The mean number of granuloma/Crohn’s disease section was 4 (SD 7), with no difference in granuloma count between the small and large bowel sections. Interestingly, the four sections positive for IS900 all contained granuloma, with a mean count of 6 granuloma/section. Crohn’s disease sections containing granulomata were not significantly more likely to amplify IS900 than those Crohn’s disease sections free of granuloma (p=0.15, Fisher’s exact test). Crohn’s disease tissues containing granulomata, how-

TABLE II  Tissues positive for TB 600 (65 kD gene) and IS900 (M paratuberculosis specific)

<table>
<thead>
<tr>
<th></th>
<th>No of samples</th>
<th>No of small bowel</th>
<th>No of large bowel</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>positive/total</td>
<td>no of samples</td>
<td>no of samples</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TB600</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crohn’s disease</td>
<td>11/31</td>
<td>4/14</td>
<td>7/16</td>
</tr>
<tr>
<td>All</td>
<td>9/20</td>
<td>3/7</td>
<td>6/12</td>
</tr>
<tr>
<td>With granulomata</td>
<td>1/10</td>
<td>0/3</td>
<td>1/7</td>
</tr>
<tr>
<td>Ulcerative colitis</td>
<td>8/20</td>
<td>6/13</td>
<td>2/6</td>
</tr>
<tr>
<td>Controls</td>
<td>4/31</td>
<td>0/14</td>
<td>4/16</td>
</tr>
<tr>
<td>IS900</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crohn’s disease</td>
<td>4/20*</td>
<td>0/7</td>
<td>4/12</td>
</tr>
<tr>
<td>All</td>
<td>0/10</td>
<td>0/5</td>
<td>0/7</td>
</tr>
<tr>
<td>With granulomata</td>
<td>0/20</td>
<td>0/13</td>
<td>0/6</td>
</tr>
<tr>
<td>Ulcerative colitis</td>
<td>0/20</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*p=0.02 for comparison of Crohn’s disease granulomatosus tissues with all controls (ulcerative colitis plus control tissues) for IS900 positivity (Fisher’s exact test). The two anal skin tags are not included in this Table; both were polymerase chain reaction negative and the Crohn’s disease derived sample contained granulomata.

Figure 2: Southern blot of Crohn’s and control tissue derived DNA amplified with IS900 primers for M. paratuberculosis. MWM = original magnification x 174 molecular weight marker digested with Hae III. Lane 1 = 1 x 10^4 dilution of Johne’s tissue in human gut DNA extract. Positive signals are in lanes 12, 15, 21, and 27, which contain Crohn’s disease tissue.
ever, were significantly more likely (p=0·02, Fisher’s exact test) to amplify IS900 on PCR when compared with all non-Crohn’s disease controls. No correlation was seen between age, absolute granuloma count, and IS900 positivity. Acid fast bacilli were not seen in any Crohn’s disease tissue section.

Discussion

We believe this is the first report of the use PCR to investigate the presence of general and specific mycobacterial DNA in Crohn’s disease and its association with the histological features of the disease, in a double blinded study. Our results suggest that only a few (13%) of all Crohn’s disease sections studied contain \textit{M. paratuberculosis} DNA, but 20% of those containing granulomata do so. All these were large bowel sections, and none showed acid fast bacilli on Ziehl-Neelsen staining. Granulomatous Crohn’s disease tissues were significantly more likely to contain \textit{M. paratuberculosis} DNA than non-Crohn’s disease tissue controls (p=0·02). We have confirmed previous reports of the wide prevalence of mycobacterial DNA in both small and large bowel tissues, and shown that, in this small study, Crohn’s disease tissues are no more likely to contain non-specific mycobacterial DNA than controls. There are a number of reasons why Crohn’s disease tissues with granulomata will possibly contain more \textit{M. paratuberculosis} DNA. The most obvious would be that this organism causes Crohn’s disease in a subgroup (20%) of patients with this histopathological appearance. Other possibilities are that granulomatous tissue is more likely to develop infection with this organism but is not caused by it, and that even the granulomas themselves may be an epiphenomenon, unrelated to the aetiology of Crohn’s disease. Of particular interest is an elegant study by Wakefield \textit{et al.}\cite{16} suggesting that most granulomas in Crohn’s disease form within walls of blood vessels. These authors speculate that the intestinal microvasculature, containing these granulomata, may contain ‘an early element in the pathogenesis of Crohn’s disease’. Our results suggest that the early element in some cases may be \textit{M. paratuberculosis} infection. One of our sections contained at least 2000 genomes of \textit{M. paratuberculosis} DNA, leading us to believe that the organism may exist in concentrated foci within some granuloma. We found no significant difference between granuloma count per Crohn’s tissue section and IS900 positivity, but there was a trend toward higher mean values in our small group of four positive samples. Sampling techniques such as ours, using small specimens, may miss a region of concentrated \textit{M. paratuberculosis} organisms. This may explain the discrepancy between our result and the result described by Sanderson \textit{et al.}\cite{17} showing \textit{M. paratuberculosis} DNA in 65% of Crohn’s disease patients. They extracted DNA from homogenates of entire surgical resections, and subsequently diluted it before PCR. Thus although they added equivalent amounts of DNA to each PCR as we did, it was extracted from a larger volume of tissue and might have a greater chance of sampling a discrete focus of infection. The use of total homogenates, however, precludes the localisation of pathogenic DNA to the specific lesions of Crohn’s disease. It is interesting that acid fast bacilli were not seen in even our most positive section, which contained at least 2000 genomes. This implies that the organisms are not acid fast and therefore exist in a cell-wall deficient form.

Although in our study we detect \textit{M. paratuberculosis} DNA in only a few Crohn’s disease specimens we believe that it is significant to the aetiology of Crohn’s disease for the following reasons. \textit{M. paratuberculosis} has been isolated from only two conditions: John’s disease (chronic enteritis) in animals and Crohn’s disease (chronic enteritis) in humans. In our studies \textit{M. paratuberculosis} DNA has only been detected in cultures from Crohn’s disease\cite{20,21} or tissue from Crohn’s disease. Of all the pathogens that have been associated with Crohn’s disease in the 80 years since its first description, \textit{M. paratuberculosis} is the only one capable of causing a disease pathologically indistinguishable from Crohn’s disease in experimental animals and also in primates.\cite{22,23} We have in this study shown that \textit{M. paratuberculosis} DNA is associated with the lesions of Crohn’s disease. Further studies are required to establish if the comparatively low yield of positive samples is a result of a low percentage of patients infected with this pathogen or a consequence of our sampling technique. As we have previously argued,\cite{24} however, if a single aetiological agent can be identified as the cause of the disease in even a few patients, this will greatly advance our understanding of the pathology of all cases of Crohn’s disease. In addition, the techniques described here could identify a subgroup of Crohn’s patients that may respond to anti-mycobacterial chemotherapy. These findings may at last advance us from a position essentially the same as Dalziel’s, when he first described Crohn’s disease 80 years ago.\cite{25} I can only regret that the etiology of the condition remains in obscurity, but I trust that ere long further consideration will clear up the difficulty.\cite{26}

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15 Rosenberg WM, Bell JI. Mycobacterium paratuberculosis DNA cannot be detected in Crohn's disease tissues. Gastroenterology 1991; 100: A611.
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