Search for *Mycobacterium paratuberculosis* DNA in orofacial granulomatosis and oral Crohn's disease tissue by polymerase chain reaction

M P Riggio, J Gibson, A Lennon, D Wray, D G MacDonald

Abstract

***Background*—Although intestinal Crohn's disease has long been suspected to have a mycobacterial cause, possible mycobacterial involvement in orofacial granulomatosis (OFG) and oral lesions of Crohn's disease has not yet been investigated.**

***Aims*—As the slow growing *Mycobacterium paratuberculosis* has been implicated in the aetiology of intestinal Crohn's disease, the potential involvement of this mycobacterial species in OFG and oral lesions of Crohn's disease was investigated.**

***Patients*—To attempt detection of the organism in OFG and oral Crohn's disease tissue samples, a polymerase chain reaction (PCR) assay was used on archival formalin fixed, paraffin wax embedded oral tissue sections from 30 patients with OFG, seven with Crohn's disease, and 12 normal controls.**

***Methods*—The PCR assay used was based on primers targeting the 5' region of the multicopy IS900 DNA insertion element of the *M paratuberculosis* genome. In order to achieve maximum sensitivity, two rounds of PCR were carried out and amplicons confirmed by Southern blot hybridisation to a digoxigenin labelled IS900 DNA probe.**

***Results*—None of the OFG and oral lesions of Crohn's disease samples were positive for *M paratuberculosis* and all normal controls were also negative.**

***Conclusions*—These results suggest that *M paratuberculosis* may not be a major aetiological agent in OFG or oral Crohn's disease lesions, although the use of paraffin wax embedded tissue as opposed to fresh tissue as a sample source could underestimate the true prevalence of the organism.**

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Keywords: oral Crohn's disease; *Mycobacterium paratuberculosis*; orofacial granulomatosis; polymerase chain reaction

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It is now widely known that chronic granulomatous transmural inflammation of the gastrointestinal tract was recognised as a clinical entity in 1913 when Dalziel described a chronic intestinal enteritis.[1] Thereafter, in 1932 Crohn described a regional ileitis distinct from tuberculosis which came to be known as Crohn's disease.[2] It has subsequently been demonstrated that Crohn's disease can affect any part of the gastrointestinal tract from mouth to anus. The first report of Crohn's disease affecting the mouth was made by Dudeney in 1969, describing a tag on the buccal mucosa of a 36 year old patient with known Crohn's disease.[3]

Oral lesions are common in patients with proven intestinal Crohn's disease and include lip swelling with and without fissuring, facial swelling, full thickness gingivitis, mucosal tags/cobblestoning, oral ulceration, and angular cheilitis.[4–6] These oral lesions may predate bowel symptoms by years.[7] Various studies have looked at the prevalence of asymptomatic intestinal Crohn's disease in patients presenting with oral lesions and this ranges from 0 to 48%.[8 9]

Orofacial granulomatosis (OFG) was a term introduced by Wiesenfeld *et al*[8] to describe the entity where patients have oral lesions resembling those of Crohn's disease clinically and histologically but do not have accompanying gastrointestinal abnormalities. The designation of OFG includes patients with granulomatous disease in whom other entities such as Crohn's disease, sarcoidosis, and tuberculosis have been excluded. Some patients with OFG may subsequently develop gut manifestations of Crohn's disease and therefore be recategorised. It has been suggested that OFG may be largely due to a type IV hypersensitivity reaction to various dietary and environmental allergens.[10]

Orofacial lesions with non-caseating granulomata on biopsy may arise in patients with and without intestinal Crohn's disease. The involvement of the mouth in granulomatous inflammatory processes holds exciting potential in terms of ease of access for biopsy and response to treatment. With this in mind, and considering the literature supporting a possible involvement of *Mycobacterium paratuberculosis* in Crohn's disease,[11–14] the possibility of *M paratuberculosis* being involved in the aetiology of OFG and oral lesions of Crohn's disease was investigated.

The polymerase chain reaction (PCR) is a highly sensitive and specific technique which has been successfully used to detect *M paratuberculosis* DNA in Crohn's disease tissue.[11–14] In the present study, PCR using primers directed against the multicopy IS900 DNA insertion element of the *M paratuberculosis* genome[15] was carried out on DNA extracted from archival paraffin wax embedded tissue sections from 30

patients with OFG, seven patients with Crohn's disease, and also 12 normal controls. This is the first study to investigate the possible presence of *M paratuberculosis* DNA in OFG and oral Crohn's disease tissue.

Patients and Methods

SELECTION OF SAMPLES

The samples used were from the collection processed and held at Glasgow Dental Hospital and School. Samples were paraffin wax sections of oral tissue, which had been examined histopathologically and classified as OFG or oral manifestations of Crohn's disease on the basis of the presence of non-caseating epithelioid cell granulomata. Thirty seven samples were obtained of which 30 were from patients with OFG and seven were from patients with known gut Crohn's disease. In each case the paraffin wax block showing the best demonstration of granulomata was selected for study. Twelve additional samples were from normal control patients. In all cases duplicate samples were obtained from the paraffin wax blocks, cut on two separate occasions.

TISSUE PROCESSING AND DNA EXTRACTION

For each sample to be analysed by PCR, five 10 µm sections were cut. The microtome knife blade was thoroughly cleaned between cutting of each different sample with xylene to prevent sample to sample contamination. The paraffin wax sections were placed in 1.5 ml centrifuge tubes and DNA was extracted using a method developed specifically for obtaining mycobacterial DNA from paraffin wax sections as previously described.[16] Briefly, tissue sections were deparaffinised in xylene, resuspended in 200 µl proteinase K (200 µg/ml)/50 mM Tris-HCl, pH 8.3, and incubated overnight at 37°C. Samples were frozen in dry ice for one minute, boiled for eight minutes, placed on ice for five minutes, and spun for two minutes to remove insoluble debris. For each PCR reaction, 40 µl of the supernatant was used.

PCR PRIMERS

The primers used for PCR (P90+ and P91+) targeted the IS900 DNA insertion element of *M paratuberculosis* as previously described,[17] and were similar to primers P90 and P91 used in another study[11] except that each primer contained an additional six or seven bases at its 5' end. The primer sequences were 5'-GAA GGGTGTTCGGGGCCGTCGCTTAGG-3' (P90+; IS900 nucleotides 15–41) and 5'-GGCGTTGAGGTCGATCGCCCACGTGA C-3' (P91+; IS900 nucleotides 427–401). The expected size of the amplification product using primer pair P90+/P91+ is 413 base pairs (bp). PCR was also used to generate an internal IS900 probe for use in subsequent Southern blot hybridisation. The sequences of the primers used for probe generation were 5'-CC AGGGACGTCGGGTATGGC-3' (P25; IS900 nucleotides 53–72) and 5'-GGTCGGCCTTA CCGGCGTCC-3' (P26; IS900 nucleotides 281–262), which give an expected amplification product of 229 bp.

PCR

PCR was carried out in a total reaction volume of 100 µl, with conditions essentially as previously described.[11 17] Each PCR consisted of 10 µl of extracted DNA and 90 µl of PCR reaction mixture comprising 1× PCR buffer (10 mM KCl, 1.5 mM $MgCl_2$, 1% Triton X-100), 2.0 units Dynazyme I DNA polymerase (Flowgen Instruments Ltd, Lichfield, UK), 0.2 mM of each of the four deoxynucleotide triphosphates and primers P90+ and P91+ each at 6 ng/µl. The primers were separated from the other components of the reaction mixture by a layer of wax (DynaWax; Flowgen Instruments Ltd). This hot start PCR method improves the specificity and yield of reaction products by preventing the reaction from starting until the wax has melted following the commencement of thermal cycling. PCR was carried out in an OmniGene thermal cycler (Hybaid Ltd, Teddington, UK). The cycling conditions comprised an initial denaturation step at 94°C for five minutes, followed by 40 cycles of denaturation at 94°C for five minutes, primer annealing at 58°C for two minutes, and extension at 72°C for three minutes, and a final extension step at 72°C for 10 minutes. A second round of PCR was then carried out using identical conditions, except that 5 µl of the first round product was used as template.

For generation of the internal 229 bp probe, PCR was set up as described above except that a $MgCl_2$ concentration of 1.0 mM and the primer pair P25/P26 were used in a single round of PCR. Target DNA was 10 ng of plasmid pPN14 which contains the cloned *M paratuberculosis* IS900 DNA insertion element. After an initial denaturation step at 94°C for five minutes, 30 cycles of denaturation at 94°C for one minute, annealing of primers at 50°C for one minute, and extension at 72°C for two minutes were carried out, followed by a final extension step at 72°C for 10 minutes. The 229 bp PCR product was purified using the Wizard PCR Preps Purification System (Promega Corporation, Southampton, UK).

SENSITIVITY OF THE PCR ASSAY

The sensitivity of the PCR assay was determined by spiking DNA extracted from paraffin wax sections of OFG, which were PCR negative for *M paratuberculosis* DNA, with serial 10-fold dilutions of *M paratuberculosis* DNA in the range 100 pg to 1 fg. PCR was carried out as described above.

PCR QUALITY CONTROL

Several anti-contamination procedures were used when carrying out PCR. Setting up of PCR reactions, thermal cycling, and post-PCR analysis of reaction products was carried out in separate rooms. Pipette filter tips were used at all stages, except when adding template DNA in which case positive displacement tips were used. Positive and negative PCR controls were included with each batch of samples being analysed; the positive control used was 1 pg of *M paratuberculosis* DNA instead of sample, and the negative control contained sterile molecular biology grade water instead of sample.

In order to serve as an internal control for the successful isolation of PCR amplifiable DNA from tissue sections, amplification of the β-haemoglobin gene was carried out for each sample analysed using nested primer PCR as previously described.[18]

AGAROSE GEL ELECTROPHORESIS

PCR reaction products were fractionated by electrophoresis of 20 µl aliquots on 2% agarose gels containing ethidium bromide (0.5 µg/ml) and visualised under ultraviolet (UV) illumination. A 100 bp DNA ladder (Pharmacia Biotech, Milton Keynes, UK) was used as a size marker.

SOUTHERN BLOT HYBRIDISATION

Amplified products were electrophoresed on 2% agarose gels as described earlier and transferred to positively charged nylon membranes (Boehringer Mannheim, Lewes, UK) by Southern blotting. Briefly, gels were prepared for blotting by soaking in denaturation solution (0.5 M NaOH/1.5 M NaCl) for 2 × 20 minutes followed by soaking in neutralisation solution (0.5 M Tris-HCl, pH 7.4/3.0 M NaCl) for 2 × 20 minutes. DNA was transferred to membranes using a capillary transfer blotting unit (Anachem Ltd, Luton, UK) with 20× SSC (3.0 M NaCl, 0.3 M sodium citrate, pH 7.0) as transfer buffer. Following transfer, membranes were rinsed in 2× SSC and DNA immobilised by exposure to an optimal dose of UV energy in a crosslinker (UVC-508; Anachem Ltd).

Membranes were hybridised overnight at 68°C with the 229 bp internal IS900 PCR product labelled with digoxigenin (DNA Labelling and Detection Kit; Boehringer Mannheim) at 25 ng/ml in standard hybridisation buffer (5× SSC, 1% blocking reagent, 0.1% N-laurylsarcosine, 0.02% sodium dodecyl sulphate (SDS)). Membranes were washed at room temperature in 2× SSC/0.1% SDS for 2 × 5 minutes, and at 68°C in 0.1 × SSC/0.1% SDS for 2 × 20 minutes. Immunological detection was carried out according to the manufacturer's instructions using an anti-digoxigenin antibody conjugated to alkaline phosphatase and colorimetric detection with 4-nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate as a colour substrate.

Results

Duplicate sets of all the samples analysed demonstrated PCR positivity for the β-haemoglobin gene, as indicated by the amplification of a 165 bp product, after two rounds of PCR using nested primer pairs (data not shown). This indicated that DNA extraction was successful for each tissue sample being analysed and that the extracted DNA was of sufficient purity and free of PCR inhibitors, thus rendering it suitable for use in subsequent PCR analysis.

The sensitivity of the PCR assay following two rounds of amplification was such that 10 fg of *M paratuberculosis* DNA was detectable by agarose gel electrophoresis (fig 1), which is the equivalent of two mycobacterial genomes.

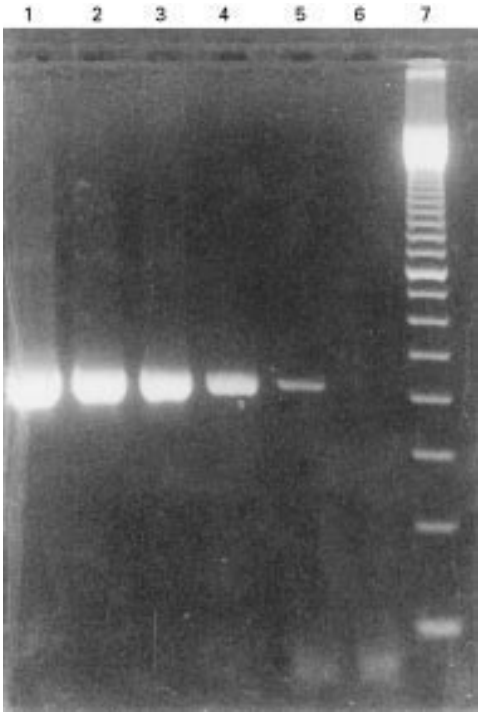

Figure 1: Agarose gel electrophoresis of PCR products (20 µl) obtained in the PCR sensitivity assay following two rounds of 40 cycles of amplification with M paratuberculosis IS900 primers P90+ and P91+. Extracted tisssue DNA from PCR negative OFG samples was spiked with serial 10-fold dilutions of M paratuberculosis DNA. Lanes 1–6, M paratuberculosis DNA at 100 pg (lane 1), 10 pg (lane 2), 1 pg (lane 3), 100 fg (lane 4), 10 fg (lane 5), 1 fg (lane 6); lane 7, 100 bp DNA ladder.

M paratuberculosis IS900 PCR was performed on duplicate sets of samples. Following a single round of 40 cycles of PCR using the *M paratuberculosis* IS900 P90+/P91+ primer pair, all of the samples were negative for the presence of *M paratuberculosis* DNA both by agarose gel electrophoresis and Southern blot hybridisation, with only the positive control producing a product of 413 bp (data not shown). In order to increase the sensitivity of the assay, a second round of PCR using identical conditions to the first round but with 5 µl of first round product as template was performed. A single OFG sample gave a PCR product, which was slightly smaller in size to that expected for *M paratuberculosis* positivity (fig 2A). However, this product did not hybridise to the 229 bp IS900 probe in Southern blot hybridisation (fig 2B). No other samples were positive by gel electrophoresis and no samples previously negative by agarose gel electrophoresis following two rounds of PCR demonstrated positivity following Southern blot hybridisation. For each batch of tissue samples being analysed, the *M paratuberculosis* positive controls were always positive and the negative controls always negative, both by agarose gel electrophoresis and Southern blot hybridisation.

Discussion

The purpose of this study was to investigate the possibility of mycobacterial involvement in OFG and oral Crohn's disease tissue samples.

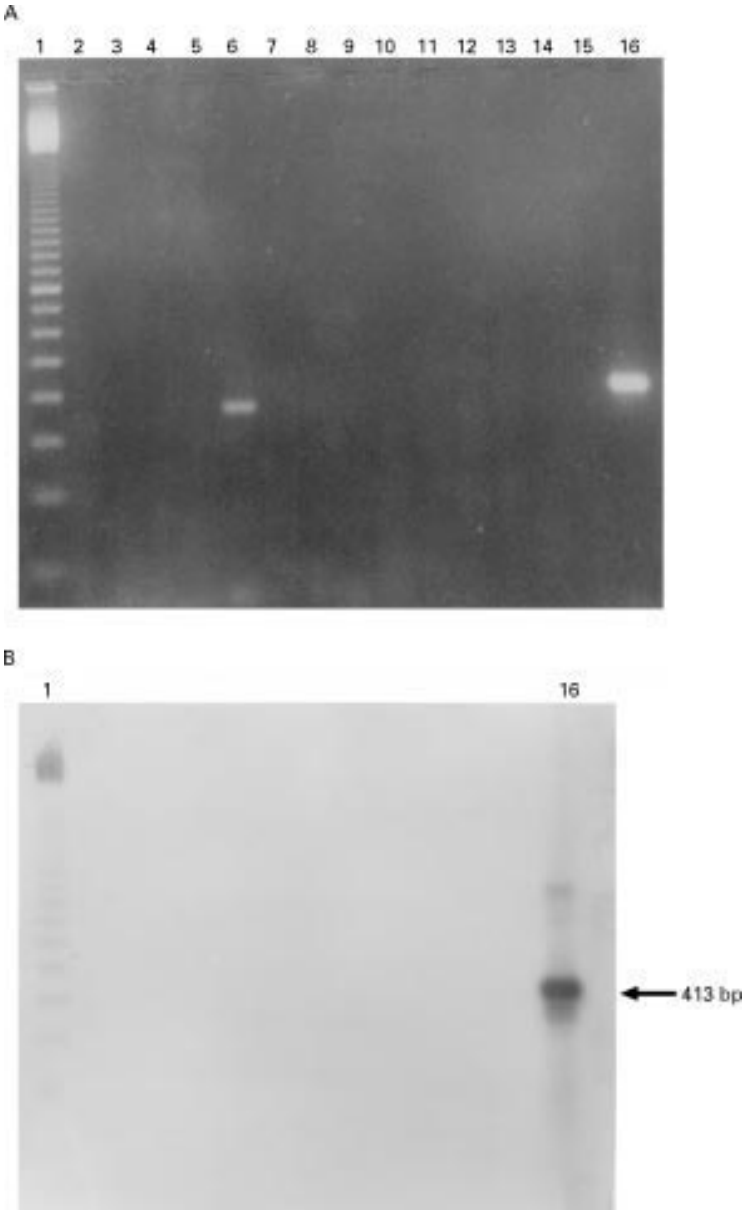

Figure 2: (A) 2% agarose gel electrophoresis of selected PCR products (20 µl) obtained from tissue DNA samples following two rounds of 40 cycles of amplification with M paratuberculosis IS900 primers P90+ and P91+. Lane 1, 100 bp DNA ladder; lanes 2–8, OFG samples; lanes 9–11, oral Crohn's disease samples; lanes 12–14, normal samples; lane 15, negative PCR control; lane 16, positive PCR control. The single PCR product obtained from the samples analysed, which is slightly smaller in size than the positive control PCR product, is shown in lane 6. (B) Corresponding Southern blot hybridisation. For orientation of the membrane, lanes 1 and 16 correspond to the 100 bp DNA ladder and PCR positive control lanes, respectively. The PCR product in lane 6 did not hybridise to the probe.

M paratuberculosis is a slow growing organism which has been shown to be the causative agent of Johne's disease,[19 20] a chronic enteritis of ruminants. Due to the extreme difficulties encountered in attempting to isolate the organism by culture, many investigators have utilised the PCR technique for its detection in diseased tissue. As *M paratuberculosis* DNA has previously been demonstrated in the intestinal tissue of up to 72% of patients affected by Crohn's disease by PCR in several studies,[11–14] it seemed prudent to investigate the possible presence of this mycobacterial species in OFG and oral Crohn's disease tissue. The primers we used for PCR in this study targeted the same 5' region of the IS900 DNA insertion element of *M paratuberculosis* as primers previously described.[11 17]

The results of our study suggest that in our patient group *M paratuberculosis* does not appear to be associated with OFG or the oral lesions of Crohn's disease. After two rounds of 40 cycles of PCR followed by Southern blot hybridisation, all tissue samples were found to be negative. The IS900 PCR assay we used was similar to that previously described by Sanderson *et al*[11] who reported that they could detect as little as 5 fg of *M paratuberculosis* DNA, which is equivalent to a single mycobacterial genome. We have demonstrated that our assay is of a similar sensitivity with the capability of detecting 10 fg of *M paratuberculosis* DNA. In view of the fact that we have used a PCR assay that is as sensitive as can reasonably be expected by carrying out two rounds of PCR and Southern blot hybridisation, and no tissue sample was positive for *M paratuberculosis* DNA it is clear that, at least in the group of patients used in this study, *M paratuberculosis* DNA is rarely found in OFG and oral Crohn's disease tissue. However, an important consideration is the possibility that some areas of infection within the tissue may be excluded when using paraffin wax sections for DNA extraction and analysis as opposed to homogenates of whole fresh tissue. In other studies, when using paraffin wax embedded tissue sections as a tissue DNA source only 7 to 13% of samples demonstrated PCR positivity for *M paratuberculosis*,[13 14] which is in sharp contrast to positivity rates of 46 to 72% obtained when using fresh tissue for analysis.[11–13] It is clearly more difficult to detect low abundance *M paratuberculosis* DNA reliably in paraffin wax samples than when using fresh tissue, as the use of fresh tissue permits the extraction of DNA from a much larger volume of tissue and consequently increases the probability of sampling a discrete focus of infection. It would be most important to extend our study by analysing both paraffin wax embedded and, where possible, fresh tissue from patients with OFG or oral Crohn's disease lesions in several geographical locations in the United Kingdom in order to investigate whether there is an altered distribution of the organism in different patient groups.

The potential involvement of *M paratuberculosis* in Crohn's disease is a controversial issue. Although some studies have demonstrated the presence of *M paratuberculosis* DNA in Crohn's disease tissue,[11–14] PCR negativity for *M paratuberculosis* DNA has been reported in other studies.[21–23] Frank and Cook[21] failed to detect *M paratuberculosis* DNA in any of 27 Crohn's disease tissue samples examined using nested PCR primers, while the use of a fluorescence PCR method demonstrated negativity in all of 68 Crohn's disease tissue samples analysed.[22] In a further study, PCR demonstrated the presence of mycobacteria with a similar frequency in the intestinal tissues of patients with Crohn's disease and of normal controls, although no *M paratuberculosis* DNA was detected in any sample.[23] PCR negativity for *M*

paratuberculosis DNA has also been obtained in tissue samples of sarcoidosis,[24] which is a generalised granulomatous disease involving multiple organs and which resembles mycobacterial infection histologically. However, the involvement of other mycobacterial species could not be excluded, particularly in view of the fact that *Mycobacterium tuberculosis* DNA was found in the bronchoalveolar lavage fluid[25] and spleens[26] of patients with sarcoidosis in other studies. The potential involvement of other mycobacterial species in OFG and oral lesions of Crohn's disease would undoubtedly be worthy of investigation.

The standard PCR protocols which have previously been used could be further refined by the use of a solid phase hybridisation capture technique, which has recently been developed and applied to PCR detection of *M paratuberculosis* and *M avium* subsp. *silvaticum*.[17] Solid phase hybridisation capture of mycobacterial DNA from tissue DNA extracts prior to PCR increases sensitivity and substantially eliminates false positives arising due to amplicon contamination. This method should prove valuable in detecting low abundance target DNA sequences in tissue samples, and its application in attempting to identify *M paratuberculosis* DNA in oral tissue may further clarify the possibility of any aetiological role for this organism in OFG and oral lesions of Crohn's disease.

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