Specific detection of *Mycobacterium paratuberculosis* by DNA hybridisation with a fragment of the insertion element IS*900*

M T Moss, E P Green, M L Tizard, Z P Malik, J Hermon-Taylor

Abstract

This paper describes the evaluation of a newly developed DNA probe for *Mycobacterium paratuberculosis*. DNA probe PCR278 is a 278 bp fragment obtained by polymerase chain reaction (PCR) amplification of the 5'-region of IS*900*, an insertion element contained in the genome of *M paratuberculosis*. This DNA probe can specifically distinguish *M paratuberculosis* from a wide range of other organisms, including members of the *M avium-M intracellulare* complex. When used in conjunction with the PCR amplification technique DNA probe PCR278 could detect as little as 10 fg (equivalent to two genomes) starting material of *M paratuberculosis* genomic DNA. Use of PCR amplification assays based on IS*900*, for the detection of *M paratuberculosis*, and homologous IS elements found in disease isolates of *M avium* should greatly help our understanding of the role of these organisms in Crohn's disease and other chronic inflammatory disorders.

A mycobacterial aetiology for Crohn's disease has long been suspected[1,2] but it is only in the last few years that mycobacteria have been isolated from Crohn's disease tissue. Burnham *et al*[3] isolated *Mycobacterium kansasii* from the lymph node of one of 27 patients with Crohn's disease; Chiodini *et al*[4] isolated a *Mycobacterium* species, subsequently shown to be *M paratuberculosis*,[5–8] from two of 11 cases of Crohn's disease but not from 3 cases of ulcerative colitis. Graham *et al*[9] isolated mycobacteria, mainly members of the *M avium-M intracellulare* and *M fortuitum* complexes, from nine of 59 cases of Crohn's disease but also isolated mycobacteria from nine of 19 cases of ulcerative colitis and from 18 of 27 non-inflammatory disease control subjects. The greatest interest has been generated by the isolation of *M paratuberculosis*. This pathogenic organism is the causative agent of Johne's disease,[10] a chronic ileitis/colitis which causes wasting disease of cattle and has many similarities for Crohn's disease.[11,12] Human isolates of *M paratuberculosis* have only been isolated from Crohn's disease patients.[13]

Determining a role, if any, for *M paratuberculosis* in Crohn's disease requires precise and sensitive probing of Crohn's disease tissue for the presence of this organism. We have recently sequenced and characterised[14] an insertion element (IS*900*), occurring 15–20 times in the genome of *M paratuberculosis*, and in this paper we report the use of a DNA probe, based on a unique portion of IS*900*, specifically to detect *M paratuberculosis*.

Department of Surgery, St George's Hospital Medical School, London SW17 0RE
M T Moss
E P Green
M L Tizard
Z P Malik
J Hermon-Taylor

Correspondence to:
Professor J Hermon-Taylor.

Accepted for publication
5 June 1990

Methods

MYCOBACTERIAL SPECIES

The 27 mycobacterial strains used in our study are given in the legend to Figure 1 and were obtained from Dr J L Stanford (University College and Middlesex School of Medicine). The original respective sources of these strains were as described previously.[15]

GROWTH CONDITIONS

Strains were grown on Lowenstein-Jensen medium at 35°C for the appropriate incubation

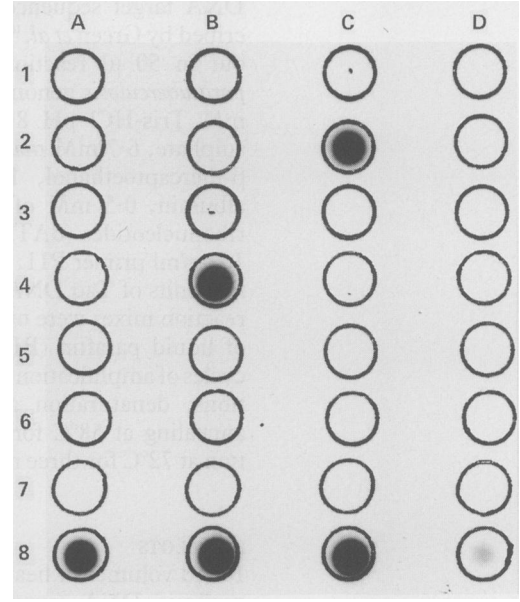

Figure 1: Dot blot hybridisation with ^{32P}-labelled PCR278. 200 ng aliquots (unless otherwise stated) of genomic DNA from 27 mycobacterial strains were dot blotted onto a nylon membrane: (1A) *Mycobacterium thermoresistibile*, (1B) *M diernhoferi*, (1C) *M bovis, BCG*, (1D) *M duvalii*; (2A) *M flavescens*, (2B) *M bovis*, (2C) *M paratuberculosis, 100 ng*, (2D) *M smegmatis*; (3A) *M intracellulare*, (3B) *M avium*, (3C) *M marinum*, (3D) *M rhodesiae*; (4A) *M neoaurum*, (4B) *M paratuberculosis, 100 ng*, (4C) *M gilvum*, (4D) *M nonchromogenicum*; (5A) *M chitae*, (5B) *M tuberculosis*, (5C) *M malmoense*, (5D) *M kansasii*; (6A) *M gastri*, (6B) *M intracellulare*, (6C) *M phlei*, (6D) *M simiae*; (7A) *M vaccae*, (7B) *M fortuitum*, (7C) *M gordonae*, (7D) *M asiaticum*; (8A) *M paratuberculosis, 200 ng*, (8B) *M paratuberculosis, 100 ng*, (8C) *M paratuberculosis, 50 ng*, (8D) *M paratuberculosis, 5 ng. After hybridisation at 65°C for 16 hours the membrane was washed with 0·1× SSC (0·15 M sodium chloride; 0·015 M sodium citrate, pH 7); 0·1% sodium dodecyl sulphate then exposed to an x ray film for three hours at −70°C.*

periods. In the case of *M paratuberculosis*, mycobactin J was present at a final concentration of 1 μg/ml.

GENOMIC DNA EXTRACTION

Cells were harvested and genomic DNA extracted in accordance with our previously described protocol.[15] Briefly, cells were lysed after sequential treatment with 100 mg/ml subtilisin (Sigma) at 37°C for 18 hours, 200 mg/ml lysozyme (Sigma) at 50°C for five hours, then 30 mg/ml pronase (Calbiochem) and 1% sodium dodecyl sulphate at 37°C for 20 hours. Two phenol/chloroform extractions were carried out and the DNA was recovered by ethanol precipitation.

PCR PRIMERS

Two oligonucleotide primers (P11 and P36) were custom synthesised for PCR amplification (Oswel DNA Service, Edinburgh). P11: 5'-CGTCGTTAATAACCATGCAG-3' and P36: 5'-GGCCGTCGCTTAGGCTTCGA-3' are found in the 5'-region of IS*900* (data not shown), are complementary to the sense and antisense strands of IS*900*, respectively, and allow amplification of a 278 bp fragment (DNA probe PCR278).

PCR CONDITIONS

Amplification of *M paratuberculosis* genomic DNA target sequences was performed as described by Green *et al.*[16] Amplification was carried out in 50 μl reaction volumes containing *M paratuberculosis* genomic DNA (10 ng-1 fg), 67 mM Tris-HCl pH 8·8, 16·6 mM ammonium sulphate, 6·7 mM magnesium chloride, 10 mM β-mercaptoethanol, 170 μg/ml bovine serum albumin, 0·5 mM of each of the four deoxyribonucleotides (dATP, dCTP, dGTP, dTTP), 10 μg/ml primer P11, 10 μg/ml primer P36, and five units of Taq DNA polymerase (Cetus). The reaction mixes were overlaid with 50 μl volumes of liquid paraffin (BDH) then subjected to 30 cycles of amplification under the following conditions: denaturation at 93°C for one minute; annealing at 58°C for one minute; polymerisation at 72°C for three minutes.

DOT BLOTS

100 μl volumes of heat denatured mycobacterial genomic DNA samples (200 ng-50 pg) were spotted onto nylon membranes (Hybond-N; Amersham) with the aid of a Bio-Dot unit (Bio-Rad). Membranes were washed in 2×SSC (1× SSC consists of 0·15 M sodium chloride; 0·015 M sodium citrate, pH 7), air dried then ultraviolet illuminated for three minutes to allow cross linking of the DNA to the membrane.

HYBRIDISATION

The membranes were prehybridised at 65°C in buffer consisting of 3×SSC; 0·1% (w/v) bovine serum albumin (fraction V; Sigma); 0·1% (w/v) ficoll (Pharmacia); 0·1% (w/v) polyvinylpyrrolidone (Sigma); 0·5% (w/v) sodium dodecyl sulphate (SDS) (Sigma); 10% (w/v) dextran sulphate (Pharmacia); and 100 μg/ml sheared and denatured salmon sperm DNA (Sigma) for at least one hour. ^{32P}-labelled DNA probes were prepared by the random hexanucleotide priming technique of Feinberg and Vogelstein.[17] After denaturation by boiling DNA probes were added to the prehybridised membranes, together with fresh hybridisation buffer. Hybridisation was carried out at 65°C overnight. Membranes were washed at 65°C in 3×SSC; 0·1% SDS for 2×30 minutes, in 1×SSC; 0·1% SDS for 2×30 minutes, and finally in 0·1×SSC; 0·1% SDS for 2×30 minutes. Membranes were exposed to X-Omat film (Kodak) at −70°C for periods of between three and 16 hours.

Bacterial strains

Aeromonas hydrophila Porton Down 13109	*Klebsiella pneumoniae* CI
Alcaligenes odorans NCTC 10388	*Lactobacillus rhamnosus* NCIB 8010
Arthrobacter globiformis NCIB 8907	*Listeria monocytogenes* CI
Bacillus subtilis CI	*Nocardia asteroides* ATCC 3318
Bacteroides asaccharolyticus NCTC 9337	*Nocardia brasiliensis* IMRV 800
Bacteroides fragilis NCTC 8343	*Peptococcus asaccharolyticus* NCTC 11461
Brevibacterium lipolyticum IAM 1413	*Peptostreptococcus magnus* CI
Candida albicans CI	*Propionibacterium acnes* VPI 0162
Cellulomonas biazotea NCIB 8077	*Propionibacterium avidum* VPI 0589
Cellulomonas fimi NCTC 7547	*Proteus mirabilis* CI
Campylobacter jejuni CI	*Pseudomonas aeruginosa* NCTC 10662
Clostridium difficile CI	*Rhodococcus corallinus* NCTC 10668
Clostridium perfringens CI	*Rhodococcus* species NCIB 9784
Clostridium septicum CI	*Rothia dentocariosa* NCTC 10207
Corynebacterium JK NCTC 11915	*Salmonella enteritidis* CI
Corynebacterium minutissimum NCTC 10284	*Staphylococcus aureus* NCTC 6571
Enterobacter cloacae CI	*Staphylococcus epidermidis* CI
Escherichia coli NCTC 10418	*Streptococcus faecalis* CI
Eubacterium aerofaciens NCTC 11838	*Streptococcus milleri* CI
Eubacterium lentum NCTC 11813	*Streptococcus pneumoniae* CI
Fusobacterium varium NCTC 10560	*Streptomyces coelicolor* A3 (2)
Gardinerella vaginalis CI	*Wolinella recta* NCTC 11489
Helicobacter pylori CI	*Yersinia enterocolitica* CI

CI=clinical isolate.

Results

SPECIFICITY OF DNA PROBE PCR278

PCR amplification of *M paratuberculosis* genomic DNA with the oligonucleotide primers P11 and P36 produces an amplification product of 278 bp (PCR278). To test the specificity of product PCR278 as a DNA probe, a dot blot containing genomic DNA from 27 mycobacterial strains was prepared. After hybridisation of this dot blot with ^{32P}-labelled PCR278 positive signals were obtained only with *M paratuberculosis*, as seen in Figure 1. A second dot blot, containing genomic DNA from a further 46 bacterial strains (Table), was similarly prepared and probed with ^{32P}-labelled PCR278. These strains were chosen on the basis of being common gut commensals or for having previously been implicated in Crohn's disease. No positive signals were observed on this dot blot (data not shown). The results from these two dot blots confirm that DNA probe PCR278 can specifically identify *M paratuberculosis* from among 27 different mycobacterial strains and from a wide range of other diverse organisms.

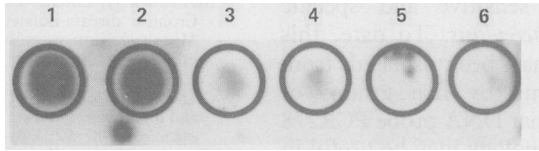

Figure 2: Testing the sensitivity of DNA probe PCR278. Dilutions of M paratuberculosis genomic DNA were prepared and spotted onto a nylon membrane: (1) 10 ng, (2) 5 ng, (3) 1 ng, (4) 500 pg, (5) 100 pg, and (6) 50 pg. After hybridisation at 65°C for 16 hours with ^{32P}-labelled PCR278 the membrane was washed with 3× SSC (1× SSC: 0·15 M sodium chloride; 0·015 M sodium citrate, pH 7); 0·1% sodium dodecyl sulphate, then exposed to to an x ray film for 16 hours at −70°C.

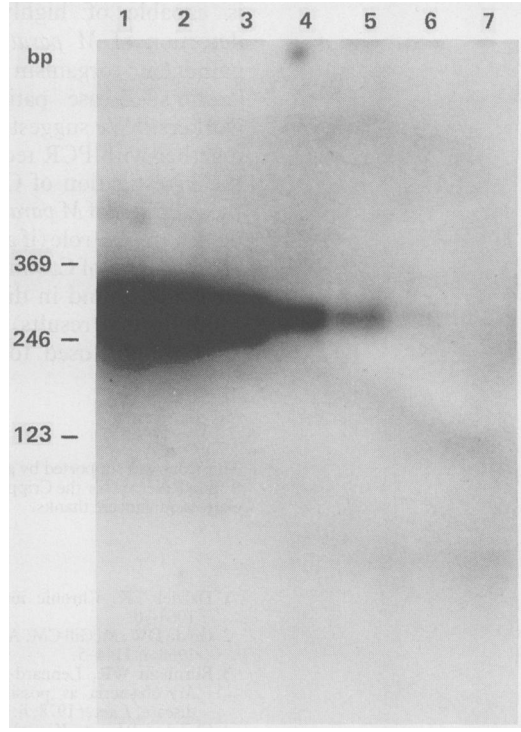

Figure 3: Southern blot analysis of PCR amplification products with labelled PCR278. Each lane contains one tenth of the PCR reaction mix. The original starting material in each PCR reaction was as follows: lane (1) 1 ng, (2) 100 pg, (3) 10 pg, (4) 1 pg, (5) 100 fg, (6) 10 fg, (7) 1 fg. Hybridisation with PCR278 was at 65°C for 16 hours and exposure of the x ray film was for 16 hours at −70°C.

SENSITIVITY OF DNA PROBE PCR278

A dot blot containing dilutions of *M paratuberculosis* genomic DNA, ranging from 10 ng to 50 pg, was prepared. Hybridisation of this dot blot with labelled PCR278 resulted in the detection of positive signals with as little as 500 pg of *M paratuberculosis* genomic DNA (Fig 2). McFadden *et al*[7] calculated the genome size of *M paratuberculosis* as $3{\cdot}1\times10^9$ Da, correspondingly the detection of 500 pg of *M paratuberculosis* genomic DNA target sequence represents the detection of 1×10^5 genomes.

In an attempt to increase further the sensitivity of DNA probe PCR278 seven aliquots of *M paratuberculosis* genomic DNA (1 ng-1 fg) were subjected to PCR amplification using primers P11 and P36. A 10 µl aliquot of each PCR reaction was subsequently electrophoresed through a 2% agarose gel. Ultraviolet visualisation of the ethidium bromide (EtBr) stained agarose gel showed a band of the correct size (278 bp), amplified from as little as 1 pg of starting material (data not shown). Sensitivity was further improved by Southern blotting[18] this gel and probing the resultant membrane with ^{32P}-labelled DNA probe PCR278 (Fig 3). The amplified 278 bp band was detected in lanes 1–6 (1 ng-10 fg). With Southern blotting the limit of detection was increased 100-fold over that achieved by ultraviolet visualisation of an EtBr stained gel. This shows that DNA probe PCR278 can, in conjunction with the PCR amplification technique, detect as little as 10 fg starting material (Fig 3; lane 6) of *M paratuberculosis* genomic DNA, the equivalent of two genomes.

Discussion

The 1451 bp nucleotide sequence of IS*900*, an insertion sequence element repeated 15–20 times within the genome of *M paratuberculosis*, has recently been determined.[14] Comparison of DNA databases to IS*900* showed an overall homology of 52% with IS*110*, an insertion sequence element identified in *Streptomyces coelicolor*.[19 20] No homologies with other insertion sequences were observed. The strongest homology between IS*900* and IS*110* occurs at their 3′-ends, at both the DNA and the amino acid level. Evidence for homologies between IS*900* and a class of related insertion elements found in disease isolates of *M avium* has been shown by Southern blot analysis (McFadden *et al*, unpublished results). Accordingly, we have tested DNA fragments originating from the 5′-end of IS*900* for specificity for *M paratuberculosis*. Unfortunately, the restriction site profile of IS*900* (data not shown) does not allow easy isolation of a suitable 5′-end fragment; consequently we have used PCR to isolate appropriate portions of the IS*900* sequence. The two oligonucleotide sequences selected for use as PCR amplification primers in this study (P11 and P36) span the 5′-region of IS*900* and their use results in the amplification of a 278 bp fragment. This amplification product (PCR278) was subsequently used as a DNA probe and our results show it can specifically distinguish *M paratuberculosis* from a wide range of organisms, including members of the *M avium-M intracellulare* complex. Hurley *et al*[21] have recently described the development of a DNA probe for the detection of *M paratuberculosis*; however, their probe was unable to differentiate members of the *M avium-M intracellulare* complex.

In a recent paper, Butcher *et al*[22] described their failure to detect, by Southern blotting and DNA hybridisation with cloned mycobacterial genomic DNA probes, the presence of mycobacterial sequences in Crohn's disease tissue. They concluded that the failure of their study could be accounted for by a lack of sensitivity in the techniques used; *M paratuberculosis* genomic DNA could not be detected at levels equivalent to one mycobacterial genome/100 human cells. Using the methods described in this paper, one of us (ZPM, unpublished results) detected one *M paratuberculosis* genome/2500 human cells.

PCR is a powerful technique capable of amplifying specific target DNA sequences $>10^5$-fold.[23 24] Our results suggest that DNA probe PCR278, in conjunction with PCR amplification,

is capable of highly sensitive and specific detection of *M paratuberculosis*. To date, this pathogenic organism has been isolated from Crohn's disease patients by five groups of workers.[24] We suggest that DNA probe PCR278 together with PCR technology may be useful in the investigation of Crohn's disease tissues for the presence of *M paratuberculosis* and will help to determine the role (if any) of this organism in the pathogenesis of Crohn's disease. Homologous IS elements, found in disease isolates of *M avium* (unpublished results) are being sequenced and may also be used to assess the role of these organisms.

This work was supported by grants from the Wellcome Trust and Action Research for the Crippled Child to whom we would like to express our sincere thanks.

1 Dalziel TK. Chronic interstitial enteritis. *BMJ* 1913; ii: 1068-70.
2 Golde DW, McGill CM. Aetiology of regional enteritis. *Lancet* 1968; i: 1144–5.
3 Burnham WR, Lennard-Jones JE, Stanford JL, Bird RG. Mycobacteria as possible cause of inflammatory bowel disease. *Lancet* 1978; ii: 693–6.
4 Chiodini RJ, van Kruiningen HJ, Thayer WR, Merkal RS, Coutu JA. Possible role of mycobacteria in inflammatory bowel disease. I. An unclassified Mycobacterium species isolated from patients with Crohn's disease. *Dig Dis Sci* 1984; **29:** 1073–9.
5 Chiodini RJ, van Kruiningen HJ, Merkal RS, Thayer WR, Coutu JA. Characteristics of an unclassified Mycobacterium species isolated from patients with Crohn's disease. *J Clin Microbiol* 1984; **20:** 966–71.
6 Chiodini RJ, van Kruiningen HJ, Thayer WR, Coutu JA. The spheroplastic phase of mycobacteria isolated from patients with Crohn's disease. *J Clin Microbiol* 1986; **24:** 357–63.
7 McFadden JJ, Butcher PD, Chiodini RJ, Hermon-Taylor J. Determination of genome size and DNA homology between an unclassified Mycobacterium species isolated from patients with Crohn's disease and other mycobacteria. *J Gen Microbiol* 1987; **133:** 211-4.
8 McFadden JJ, Butcher PD, Chiodini RJ, Hermon-Taylor J. Crohn's disease-isolated mycobacteria are identical to Mycobacterium paratuberculosis, as determined by DNA probes that distinguish between mycobacterial species. *J Clin Microbiol* 1987; **25:** 796–801.
9 Graham DY, Markesich DC, Yoshimura HH. Mycobacteria and inflammatory bowel disease. Results of culture. *Gastroenterology* 1987; **92:** 436–42.
10 Chiodini RJ, van Kruiningen HJ, Merkal RS. Ruminant paratuberculosis (Johne's disease): the current status and future prospects. *Cornell Vet* 1984; **74:** 218–62.
11 Patterson DSP, Allen WM. Chronic mycobacterial enteritis in ruminants as a model for Crohn's disease. *Proceedings of the Royal Society of Medicine* 1972; **65:** 998–1001.
12 Morgan KL. Johne's and Crohn's. Chronic inflammatory diseases of infectious aetiology? *Lancet* 1987; i: 1017–9.
13 Hampson SJ, Portaels F, Thompson J, *et al.* DNA probes demonstrate a single highly conserved strain of Mycobacterium avium infecting AIDS patients. *Lancet* 1989; i: 65–8.
14 Green EP, Tizard MLV, Moss MT, *et al.* Sequence and characteristics of IS900, an insertion element identified in a human Crohn's disease isolate of Mycobacterium paratuberculosis. *Nucleic Acids Res* 1989; **17:** 9063–73.
15 Visuvanathan S, Moss MT, Thompson J, *et al.* Simple enzymic method for isolation of DNA from diverse bacteria. *J Microbiol Methods* 1989; **10:** 59–64.
16 Green PM, Bentley DR, Mibasham S, Nilsson IM, Gianelli F. Molecular pathology of haemophilia B. *EMBO J* 1989; **8:** 1067–72.
17 Feinberg AP, Vogelstein B. A technique for radiolabelling DNA restriction fragments to high specific activity. *Anal Biochem* 1984; **137:** 226–7.
18 Southern EM. Detection of specific sequences amongst DNA fragments separated by gel electrophoresis. *J Mol Biol* 1975; **908:** 503–17.
19 Chater KF, Bruton CJ, Foster SG, Tobek I. Physical and genetic analysis of IS110, a transposable element of Streptomyces coelicolor A3(2). *Mol Gen Genet* 1985; **200:** 235–9.
20 Bruton CJ, Chater KF. Nucleotide sequence of IS110, an insertion sequence of Streptomyces coelicolor A3(2). *Nucleic Acids Res* 1987; **15:** 7053–65.
21 Hurley SS, Splitter GA, Welch RA. Development of a diagnostic test for Johne's disease using a DNA hybridization probe. *J Clin Microbiol* 1989; **27:** 1582–7.
22 Butcher PD, McFadden JJ, Hermon-Taylor J. Investigation of mycobacteria in Crohn's disease tissue by Southern blotting and DNA hybridization with cloned mycobacterial genomic DNA probes from a Crohn's disease isolated mycobacteria. *Gut* 1988; **29:** 1222–8.
23 Saiki RK, Scharf S, Faloona F, *et al.* Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* 1985; **230:** 1350–4.
24 Chiodini RJ. Crohn's disease and the mycobacterioses: a review and comparison of two disease entities. *Clin Microbiol Rev* 1989; **2:** 90–117.