Use of polymerase chain reaction for early identification of *Mycobacterium tuberculosis* in positive cultures

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Abstract

Aims: **To develop a readily applicable polymerase chain reaction (PCR) based technique which would permit the identification of *Mycobacterium tuberculosis* complex isolates from Bactec phials at an earlier stage than currently available methods.**

Methods: **Mycobacterial cells cultured in Bactec 12B medium were harvested by centrifugation. The cells were lysed by heating in distilled water. Oligonucleotide primers based on the sequence of the gene coding for the immunogenic protein MPB64 were then used to amplify a 240 base pair fragment of DNA directly from the crude cell lysate. The PCR product was visualised under ultraviolet light following electrophoresis of an aliquot in an agarose gel containing ethidium bromide. The sensitivity of the PCR was adjusted so that about 600 cfu of *M tuberculosis* gave a positive result. The lowest growth index at which this method of identification might be applied to Bactec phials was determined and a number of routine cultures giving a positive growth index examined.**

Results: ***M tuberculosis*** **was positively identified at the lowest growth index, as determined by the Bactec system. Of 45 routine cultures examined, with growth indexes ranging from 6 to 999, the 15 confirmed by conventional means to contain *M tuberculosis* were correctly identified from 1 ml of culture medium.**

Conclusions: **The method described can be used to identify *M tuberculosis* isolates cultured in the Bactec system at the earliest detectable rise in growth index. It may therefore allow cultured mycobacteria to be identified at an earlier stage than conventional methods or the commercially available DNA probes adapted for use with the Bactec system.**

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The rapid and specific detection of *Mycobacterium tuberculosis* in clinical samples remains difficult despite years of research. Examination of smears stained by the Ziehl-Neelsen or fluorochrome methods remain the only widely available methods which provide rapid evidence of mycobacterial infection. The need for a large number of organisms in a sample (estimated at 1000/ml for sputum)[1] limits the value of this technique. Furthermore, as mycobacteria other than tuberculosis (MOTT) are increasingly recognised as pathogens, particularly in the immunocompromised, species level identification is now more urgent.

Culture is much more sensitive than direct smear examination and provides definitive identification. Culture is slow, however, requiring three to six weeks using conventional methods, though somewhat less using the Bactec radiometric growth detection system.[2]

Identification of mycobacteria grown in Bactec cultures is based on selective inhibition of certain species. Selective inhibition of the *M tuberculosis* complex of organisms by NAP (p-nitro-a-acetylamino-hydroxypropiophenone) is the manufacturer's recommended procedure. Identification in this way requires an initial growth index of 50 to 100 and may require six days for an interpretable result.[3]

The early identification of mycobacterial species in positive Bactec cultures using an enzyme linked immunoassay method has been described,[4] but some cross-reactivity between *M tuberculosis* and *M avium* occurred. Others have used radioactive DNA probes for the rapid species level identification of mycobacterial isolates from Bactec cultures.[5] More recently, non-radioactive probes (AccuProbe) have become available, but a dense culture of mycobacteria (equivalent to McFarland No 1) is required. We wanted to develop a simple non-radioactive method to permit early identification of *Mycobacterium tuberculosis* complex isolates cultured in Bactec phials. It has been shown that *M bovis* cells can be lysed by exposure to proteinase K and that following heat denaturation of the proteinase K DNA amplification from the crude cell lysate was easily achieved.[6] We considered that using the specific primers described by Shankar *et al*[7 8] amplification from crude cell lysate could provide a simple method for the identification of *M tuberculosis* in positive Bactec cultures at an early stage. These primers amplify a 240 base pair fragment from the gene encoding the immunogenic protein MPB64 cloned from *M bovis BCG*.[9]

Methods

The following strains were obtained from the Central Public Health Laboratory of the Public Health Laboratory Service: *M tuberculosis H37Rv* NCTC 07416; *M bovis* NCTC 10772; *M bovis BCG* NCTC 5692; *M microti* NCTC 8710; *M avium* NCTC 08559; *M intracellulare* NCTC 10425; *M scrofulaceum* NCTC 10803; *M kansasii* NCTC 10268; *M phlei* NCTC 08151; *M gordonae* NCTC 10267; *M terrae* NCTC 10856; *M xenopi* NCTC 10042; *M*

szulgai NCTC 10831; *M fortuitum* NCTC 2006; *Nocardiae species* NCTC 434; and *Actinomyces bovis* NCTC 45000. Strains of *Pseudomonas aeruginosa, Escherichia coli, Haemophilus influenzae, Streptococcus pneumoniae* and *Corynebacterium diptheriae* var *mitis* were locally maintained derivatives of type cultures. The candida species was a recent clinical isolate.

The clinical specimens used were routine specimens submitted to this laboratory for examination for *M tuberculosis* and were mainly sputum samples.

Mycobacterial species were cultured in Bactec 7H12 phials (Becton Dickinson) at 37°C and read daily on a Bactec 460 instrument (Becton Dickinson) until a uniform growth index of 999 was reached. Non-mycobacterial species were cultured in brain heart infusion broth (Oxoid) or chocolate blood agar plates (for *H influenzae*).

Aliquots of each culture were stored at −70°C. Mycobacterial cells were harvested from 0·2 ml of culture by centrifugation at 16 000 × *g* for 15 minutes in a desk top centrifuge. For non-mycobacterial cells a variable volume of culture was used to yield a pellet of 10^6 to 10^7 cells (larger numbers of cells result in inhibition of the amplification process). The supernatant was discarded and the pellet resuspended in 50 μl of sterile distilled water. This suspension was then transferred to a Gene-Amp tube (Perkin Elmer) and overlaid with light mineral oil (Perkin Elmer). Cells were lysed by heating to 95°C for 20 minutes.

The oligonucleotide primers 5'TCCG-CTGCCAGTCGGCTTCC-3' (MPB64-1) and 5'GTCCTCGCGAGTCTAGGCCA-3' (MPB64-2) and the confirmatory probe 5'CTTCAACCCGGGGGAGT-3' (nt 601–617) were synthesised on a DNA synthesiser (Applied Biosystems) and deprotected by the recommended procedures. A 2 × reaction mix was prepared (100 mM KCl, 20 mM TRIS-HCl (pH 9·0) at room temperature, 3·0 mM MgCl, 0·02% gelatin (w/v), 0·2% Triton X-100, 400 μM of each of dATP, dTTP, dCTP, and dGTP) with 50 ng of each primer per 50 μl. To each 50 μl of crude cell lysate we added 50 μl of the 2 × reaction mix, resulting in a final reaction volume of 100 μl. After preheating the 100 μl to 95°C for five minutes 2·5 U of *Taq* DNA polymerase (Promega) was added and 32 or 36 cycles of amplification were performed in a DNA thermal cycler (Perkin Elmer). The time temperature profile was 94°C for 30 seconds, 60°C for 30 and 72°C for 60 seconds. The reaction products were visualised under ultraviolet light following electrophoresis of 10 μl aliquots of the reaction product in ethidium bromide stained 3·5% Nu-Sieve agarose gels (FMC).

Initially the identity of the reaction product was confirmed by DNA probe hybridisation. The DNA was transferred from the gel on to a Nytran membrane overnight, according to the method of Southern[10] and the DNA fixed to the membrane by exposure to ultraviolet light. The membrane was then probed with the confirmatory oligonucleotide probe end labelled with y-^{32}P-ATP, as described elsewhere.[6] Autoradiography was performed by exposure of the membrane to *x*-ray film (X-AR5, Kodak) at −80°C for five hours.

To determine if the target of amplification was conserved in wild strains of *M tuberculosis* and *M bovis*, 42 strains of the former and five of the latter were examined by PCR.

The criterion for a positive PCR assay in the following experiments was a visible amplified product of 240 base pairs following electrophoresis of a 10 μl aliquot of reaction product in an agarose gel. To determine the number of cfu detectable by this method a dense culture of *M tuberculosis H37Rv* was homogenised by agitation with glass beads and metal clips on a magnetic stirrer. A series of 10-fold dilutions was prepared in Bactec 7H12 medium and viable counts performed using the method of Miles and Misra.[11] Colonies were counted following three weeks of incubation at 37°C. One ml aliquots of each dilution were subjected to PCR as outlined above.

To determine the growth index at which this method could be applied 45 Bactec phials were inoculated with 2–5 cfu each of *M tuberculosis H37Rv*. The phials in sets of three (A, B, C) were labelled 1 to 15. The Bactec phials were incubated at 37°C and read daily on a Bactec 460 instrument and the growth index recorded. The growth index for each day was taken as the mean growth index of all the phials read on that day. Zero growth index was taken as the mean of 10 uninoculated phials. Each day 1 ml aliquots were stored from each of the three phials predesignated for that day at −70°C for subsequent examination by PCR.

Finally, 1 ml aliquots from a series of routine Bactec cultures were examined by PCR to determine if the method could be applied to actual clinical samples. Bactec bottles were inoculated with digested, decontaminated, and concentrated clinical specimens, according to the manufacturer's instructions. The cultures were incubated at 37°C and read twice weekly for three weeks and then finally at seven weeks before discarding. At the earliest detectable rise in growth index 0·1 ml was inoculated on to a blood agar plate which was incubated at 37°C overnight. Only samples yielding no growth on blood agar were examined by PCR. A total of 45 specimens were examined by PCR with growth index on examination of 6 to 999.

Results

SPECIFICITY OF THE PRIMERS

The specificity of the primers for organisms of the *M tuberculosis* complex (*M tuberculosis, M bovis, M bovis BCG, M microti*) was confirmed under the conditions outlined in methods (fig 1). The primers did not amplify DNA from any of 10 species of MOTT nor from organisms of related genera (*Actinomyces, Nocardia, Corynebacteria*), from common respiratory pathogens (*S pneumoniae, H influenzae*), nor from yeast or Gram negative bacilli which may contaminate Bactec phials. Failure of the MOTT and other organisms to amplify was

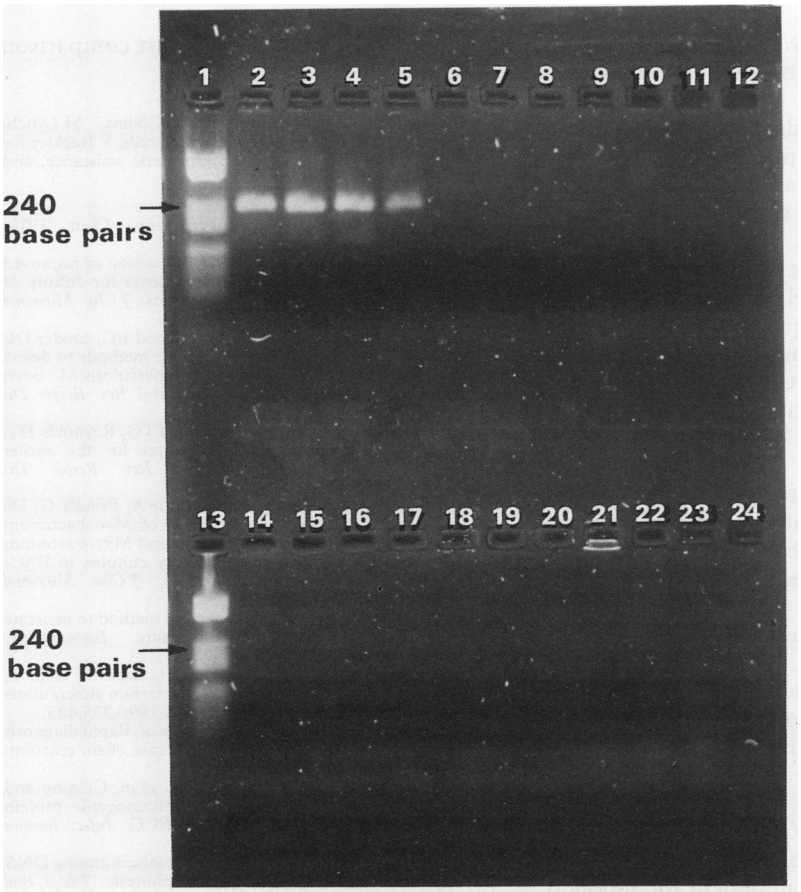

Figure 1 Specificity of primers MPB64–1 and 2. Electrophoresis of a 10 µl aliquot from each reaction in 3·5% Nu-Sieve agarose gel. Lanes 1 and 13, Hae111 digested pBR322 (size standard). Lane 2, M tuberculosis H37Rv; 3, M bovis; 4, M bovis BCG; 5, M microti; 6, M intracellulare; 7, M avium; 8, M scrofulaceum; 9, M kansasii; 10, M gordonae; 11, M terrae; 12, M xenopi; 14, M phlei; 15, M szulgai; 16, M fortuitum; 17, Actinomyces bovis; 18, Nocardia species; 19, Corynebacterium diptheriae; 20, Candida; 21, Ps aeruginosa; 22, H influenzae; 23, S pneumoniae; 24, E coli.

not due to inhibition of the amplification process by the crude cell lysate or to failure of lysis, and this was confirmed by successful amplification from all but two of the organisms, under identical conditions, using primers based on the sequence of highly conserved regions of the gene coding for 16SrRNA[6] (data not shown).

DNA probing of the Southern blot confirmed the nature of the amplified products, but as the PCR reaction is entirely specific, probe confirmation was dispensed with thereafter.

CONSERVATION OF TARGET OF AMPLIFICATION

All 47 wild strains of *M tuberculosis* complex examined by PCR contained the target of amplification as evidenced by the characteristic product at 240 base pairs, suggesting that the target of amplification is highly conserved.

Results of PCR assay days 1 to 15 on each set of three phials

	Day														
Phial	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*
A	–	–	–	–	–	–	–	–	–	+	+	+	+	+	+
B	–	–	–	–	–	–	+	–	–	+	+	+	+	+	+
C	–	–	–	–	–	–	–	–	–	+	+	+	+	+	+

Three phials examined each day (A, B, and C).
+ = visible band at 240 base pairs on gel electrophoresis.
– = no band visible following electrophoresis.

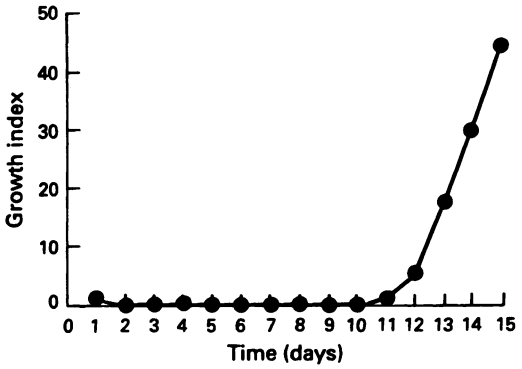

Figure 2 Mean daily growth index of inoculated Bactec phials. Mean daily growth index as measured on the Bactec 460 instrument showing a clear rise in growth index from day 12 onwards.

SENSITIVITY OF THE ASSAY

The examination of a series of 10-fold dilutions of *M tuberculosis* by PCR using the criterion of positivity outlined, gave a positive result from the 10 000-fold dilution after 32 cycles of amplification. Using 36 cycles of amplification a 100 000-fold dilution gave a positive result. Viable counts indicate that these dilutions represent, respectively, 600 cfu and 60 cfu per ml of culture medium.

Using PCR at the higher level of sensitivity false positive results can occur, even with rigorous precautions to prevent contamination. At the lower level of sensitivity false positive results due to PCR product carryover are much more readily avoided.

The performance of the assay using 32 cycles of amplification compared with detection of *M tuberculosis* by a rise in growth index was evaluated. Because of the small inoculum (2–5 cfu) 12 days were required for the growth index to show a clear rise (fig 2). The PCR assay was positive for all phials from day 10 onwards (table). The positive isolate taken on day 7 is possibly attributable to this phial having received an initially larger inoculum, despite the homogenisation of the inoculum.

CLINICAL SPECIMENS

Fifteen of the 45 positive cultures with a growth index of more than five and yielding no growth on blood agar were confirmed as containing *M tuberculosis* by standard methods. Two contained *M avium-intracellulare* and no mycobacteria were isolated from the remaining 28 cultures. All 15 cultures were positive for *M tuberculosis* complex by PCR. The remaining 30 cultures and all negative controls were negative. The growth index at the time of PCR was less than 10 for three samples and less than 50 for eight. The growth index of the remaining seven phials ranged from 51 to 999 at time of first examination by PCR.

Discussion

The capacity of PCR specifically to amplify segments of DNA has been extensively investigated for the diagnosis of infectious disease. It seems to be particularly suitable for the detection of pathogens present in small numbers, or which are difficult or impossible to culture.

Tuberculosis represents such a challenge and has stimulated several groups to investigate the application of PCR in this area.[7 8 12-18] The methods reported to date are complex and therefore not readily applicable in a diagnostic laboratory. The application of PCR to the diagnosis of infectious disease is also greatly impeded by problems with contamination, resulting in false positive results.[8] False positive results due to low level DNA contamination occur when the technique is used at very high levels of sensitivity because even a single copy of target DNA will give a positive result. We have used PCR with restricted sensitivity (thus avoiding false positive results due to PCR product carryover) to identify cultured *M tuberculosis* at an early stage.

The use of these primers for identification of cultured *M tuberculosis* has been suggested but not previously described. The application of PCR in this way requires a method comparable in simplicity with commercially available DNA probe methods.

Because of the complex nature of the mycobacterial cell wall, the consensus is that several enzymatic steps may be required to achieve cell lysis. By demonstrating that simple heating in sterile distilled water is adequate and by combining this with PCR from the resulting crude cell lysate, we believe that the method described meets this requirement for simplicity. Furthermore, it is positive at lower growth indexes than the available probes[5] and requires only a small volume of medium. Because of the specificity of the primers demonstrated by us and others, probe confirmation of the nature of the amplified product is not required.

The method described is limited by the requirement for cultivation and is therefore not a substitute for direct detection of mycobacteria in clinical samples. Currently, however, cultivation of mycobacteria remains central to the routine diagnosis of tuberculosis. We envisage the use of this method to identify mycobacteria cultured in the Bactec system at the earliest rise in growth index—that is four to five days earlier than is possible with the available DNA probes and seven to 10 days earlier than is possible with the conventional growth inhibition tests. A prospective evaluation of these methods and a cost comparison are required.

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