Multiplex PCR for identifying mycobacterial isolates

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Abstract

Aims—To develop a multiplex polymerase chain reaction (PCR) method to facilitate identification of mycobacterial isolates.

Methods—Type strains of 14 species of mycobacteria and 56 clinical isolates were lysed by boiling in TE Triton. The lysate (5 µl) was used directly in a PCR reaction incorporating three pairs of PCR primers expected to amplify fragments from the genome of (a) all mycobacteria, (b) Mycobacterium tuberculosis complex only and (c) M avium only. PCR products were visualised by electrophoresis on agarose gels.

Results—Multiplex PCR applied to 14 type strains yielded patterns on electrophoresis which permitted identification of the mycobacterial isolates as M tuberculosis complex, M avium or as mycobacteria other than the former. The identification of 56 clinical isolates by multiplex PCR was consistent with the PCR products and was accomplished in less than one working day.

Conclusions—This method may facilitate rapid and convenient identification of most clinical isolates of mycobacteria by PCR and gel electrophoresis. Further evaluation is warranted.

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Keywords: PCR, mycobacteria, identification.

The number of mycobacterial species known to cause human disease has increased considerably in recent years and considerable progress has been made in improving the speed of detection of mycobacteria using both culture and non-culture techniques. However, the processes involved in the identification of cultured mycobacteria could be improved. Rapid identification of Mycobacterium tuberculosis and M avium can be accomplished using commercially available DNA probes. At present probes are not readily available for the less common species of mycobacteria and the need to examine cultures with an extensive array of probes may prove to be expensive.

A number of general methods for the identification of mycobacteria based on interspecies genetic differences have been described. Telenti et al described a method based on interspecies variations in the sequence of the gene encoding the 65 kilodalton mycobacterial heat shock protein. Polymerase chain reaction (PCR) primers were designed to amplify a 439 base pair fragment of the gene. The sequence variation in the fragment from each species was revealed by digestion of separate aliquots of the amplified fragment by two restriction endonucleases, BstEII and HaeIII. Similar methods have been described based on other areas of the mycobacterial genome by Vanechoute et al and by Pikaytis et al.

We have previously performed an evaluation of the method of Telenti et al and found it useful. The method could be made more suitable for use in the clinical laboratory if the need to perform digestion on the PCR fragment from most isolates could be eliminated.

We and others have previously studied a primer pair MPB64-1 and MPB64-2 which amplify a 240 base pair product exclusively from members of the M tuberculosis complex (M tuberculosis, M bovis, M bovis BCG). On review of the literature, we identified a primer pair which amplified a 180 base pair fragment exclusively from M intracellulare. A multiplex PCR reaction incorporating all three primer pairs might permit identification of members of the M tuberculosis complex and M avium without the need for restriction enzyme digestion. Gel electrophoresis of the PCR products from a reaction including all three primer pairs was expected to yield two bands from M tuberculosis complex isolates (one at 439 base pairs and one at 240 base pairs), two bands from M avium isolates (one at 439 base pairs and one at 180 base pairs) and a single band from all other mycobacterial species. Those clinical isolates which are neither M tuberculosis complex nor M avium could then be identified by restriction endonuclease digestion as described previously.

Methods

CULTURE

The following type strains were obtained from the Central Public Health Laboratory, London. M tuberculosis H37Rv NCTC07416, M bovis NCTC10772, M avium NCTC8559, M intracellulare NCTC10425, M scrofulaceum NCTC10803, M fortuitum NCTC2006, M chelonae NCTC, M xenopi NCTC10042, M gordoniae NCTC10267, M terrae NCTC10856, and M szulgai NCTC10831. M kansasii, M marinum, M asiaticum, M non-chromogenicum, M phlei, and M simiae were locally maintained derivatives of type cultures. Of the clinical isolates, 36 were from our laboratory and 20 from the Mycobacterium Reference Unit, Public Health Laboratory Service, Cardiff.
PREPARATION OF MYCOBACTERIA FOR AMPLIFICATION

Mycobacteria were cultured in Bectec medium or on Lowenstein-Jensen slopes. The mycobacteria were harvested from 0.5 ml liquid culture by centrifugation and from Lowenstein-Jensen slopes with a sterile loop. The cells were suspended in 50 μl TE Triton (10 mmol Tris/HCl, pH 8.0, 1 mmol EDTA, and 1% Triton X 100) and boiled for 30 minutes to induce cell lysis. This lysate (5 μl) was used as the template in the PCR reactions.

DNA AMPLIFICATION

Oligonucleotides Tb11 (5'-ACCAACGATG-GGTGTGTCCCAT-3') and Tb12 (5'-CTTTGTCGATCAGCCTGCTTCC-3')6, MPB64-1 (5'-TCCGCCAGTCAGCTGCTTCC-3') and MPB64-2 (5'-GTTCCTGCGAGTCTAGG-3')8 were synthesised by Pharmacia (Sollentuna, Sweden). PCR was performed in 25 μl reaction volumes covered with two drops of mineral oil. The conditions were 100 mM KCl, 20 mM Tris/HCl (pH 8.0), 1.5 mM MgCl2, 0.2% Triton X 100, 200 μM each of deoxynucleotide triphosphate (dATP, dUTP, dGTP, and dCTP), 0.5 μM each of TB11 and TB12, 0.15 μM each of MPB64-1 and MPB64-2, 0.45 μM each of MYC-GEN-F and MYC-R, and 0.63 units of Taq polymerase (Promega, Madison, Wisconsin, USA). The thermal profile used was 95°C for one minute, 60°C for one minute and 72°C for one minute; 45 amplification cycles were performed. PCR products were analysed by electrophoresis of a 5 μl aliquot of the reaction on an ethidium bromide stained agarose gel. Where appropriate equal aliquots of the remaining reaction volume were digested as previously described.9,10 Standard precautions against contamination, including physical separation and use of plugged pipette tips and positive displacement tips, were taken and negative controls were included with each PCR run.

Figure 1 An ethidium bromide stained 3.5% Nu-Sieve agarose gel showing patterns obtained on electrophoresis of multiple PCR products. Lane 1, molecular weight marker V (Boehringer Mannheim, Mannheim, Germany); lane 2, Mycobacterium; lane 3, M bovis; lane 4, M avium; lane 5, M intracellulare; lane 6, M scrofulaceum; lane 7, M kansasi; lane 8, M xenopi; lane 9, M xulga; lane 10, M chelonae; lane 11, M fortuitum; lane 12, M marinum.

Figure 2 An ethidium bromide stained 3.5% Nu-Sieve agarose gel showing patterns obtained on electrophoresis of multiple PCR products. Lane 1, molecular weight marker V (Boehringer Mannheim); lane 2, M asiaticum; lane 3, M gordonae; lane 4, M terrae; lane 5, M nonchromogenicum; lane 6, M phlei; lane 7, M simiae; lane 8, mixed culture of M avium and M terrae; lane 9, mixed culture of M avium and M scrofulaceum; lane 10, 11, clinical isolates of M tuberculosis.

Results

The multiplex PCR method applied to M tuberculosis and M bovis yielded the expected species specific 240 base pair band and the mycobacterial genus specific band at 439 base pairs (fig 1). Amplification of M avium yielded a band at 180 base pairs in addition to the 439 base pair band (fig 1). M avium specific band at 180 base pairs was also detected from mixed cultures of M avium and M terrae (fig 2). A mixed culture of M avium and M tuberculosis yielded both species specific bands in addition to the common band at 439 base pairs (fig 2). The other 11 mycobacterial species including M intracellulare yielded only the genus specific band at 439 base pairs.

The series of 56 clinical isolates analysed were grouped on the basis of the multiplex PCR reaction as M avium (28 strains), M tuberculosis complex (17 strains) and other mycobacteria (11 strains). The other mycobacteria were identified as M intracellulare (n = 2), M kansasi (n = 4) and one each of M gastri, M gordonae, M xenopi, M terrae, and M marinum by the pattern obtained on digestion of the 439 base pair fragment (data not shown). Results obtained using conventional methods of identification were consistent with these results.

In some cases multiplex PCR on members of the M tuberculosis complex or M avium yielded only the species specific product at 240 or 180 base pairs, respectively. Competitive inhibition of amplification of the larger 439 base pair product may have occurred. This does not create problems in identifying the mycobacterial species present.

Discussion

In recent years considerable advances have been made in the speed and ease of identification of the many mycobacterial species now implicated in disease. Species specific DNA probes have proved useful but are not available for the full range of mycobacterial pathogens. We have previously evaluated the method described by Teleti et al11 for identification of mycobacteria but found the requirement for digestion of all PCR products inconvenient.9 The multiplex PCR
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method described here permits rapid identification of most clinical isolates. Most of the remaining isolates can be identified by digestion of the 439 base pair fragment. Under ideal circumstances, the procedure can be performed in less than one working day and only small numbers of cells are required. Further evaluation of the method is needed to determine its potential for use in the clinical laboratory and how it might be incorporated into the workload of the clinical laboratory.

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