PRELIMINARY STUDIES ON THE DETECTION OF *MYCOBACTERIUM AVIUM-INTRACELLULARE* COMPLEX USING DNA PROBE FROM A CLINICAL ISOLATE

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ABSTRACT

Genomic DNA from a clinical isolate of *Mycobacterium avium-intracellulare* complex was purified and cloned in PBR 322 at the tetracycline resistance site using Bam HI restriction enzyme. A 16 kb cloned fragment was purified, radiolabeled and used as a probe. Genomic DNA isolated from nineteen MAC strains, three *M. tuberculosis* strains and one *M. kansasii* strain were digested with Eco RI restriction enzyme, Southern blotted and hybridized with the 16 kb cloned and labeled fragment. Twelve MAC strains showed positive hybridization although five strains gave faint signals. Positive hybridization was noted in two out of the three *M. tuberculosis* strains, possibly due to shared DNA homology. No signal was received from the single *M. kansasii* strain used in this study.

KEY WORDS : *Mycobacterium avium-intracellulare* (MAC), genomic DNA (MAC), cloning MAC gene, DNA probe, Southern blotting MAC gene.

INTRODUCTION

Recombinant DNA technology developed over last 25 years has proven to be very helpful for the rapid diagnosis of diseases that require long waiting time by conventional methods. *Mycobacterium avium-intracellulare* complex (MAC) has emerged as a significant disease agent following advent of AIDS epidemic globally (1). Because of high frequency of isolation of MAC from clinical samples from AIDS patients, the increasing importance of MAC pathology in patients with AIDS and a very lengthy procedure for identification of mycobacterial species, it became necessary to search for rapid and reliable methods for diagnosis of mycobacterial infections including MAC (2-5).

Efforts are on to develop suitable diagnostic probes for MAC (2-4). However, most of the probes have met with only limited success. In addition, the

commercially available diagnostic probe for MAC is costly and difficult to procure locally. In a country like India there is a necessity for the development of cheaper probes and more so from clinical isolates in India because there could be variations in the strains from different countries and even from different regions of the same country. The present study was aimed at developing a diagnostic probe for MAC indigenously.

MATERIALS AND METHODS

MAC strains - Biochemical characterisation of nineteen strains isolated from the patients attending the Clinical Research Centre, Vallabhbhai Patel Chest Institute for treatment of pulmonary mycobacteriosis was undertaken. The strain demonstrating maximum number of positive standard biochemical tests conforming to *Mycobacterium avium-intracellulare* was taken up for preparation of genomic DNA and cloning (6).

Isolation and purification of MAC genomic DNA – The method of isolation of genomic DNA from mycobacteria was standardised earlier by Bose et

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al 1993, in this laboratory and was followed for the present study (7). Briefly, mycobacterial cells cultured in liquid broth (Middlebrook 7H9, DIFCO) were harvested at the early exponential phase and washed twice with TE (Tris- HCl pH 8.0, 10 mM; EDTA 1mM). An equal volume of saturated cesium chloride solution containing 1% Triton-X was added, the mixture was rocked gently for 10 minutes and sterile distilled water (thirty volumes) was added. The cells were centrifuged, mixed with lysozyme (10 mg/ml) and kept on ice for one hour. SDS (sodium dodecyl sulphate) and proteinase K were added before placing it in 55°C waterbath for four hours. Subsequently, guanidium isothiocyanate (GIT) cocktail (guanidium thiocyanate, Tris-HCl, EDTA, NaCl, N-lauorylsarcosine, 2-mercaptoethanol) and 1% CTAB (cetyl trimethyl ammonium bromide) were added and kept at 65°C for 30 minutes in slow speed water shaker bath. DNA was purified by phenol- chloroform extraction and ribonuclease treatment (7).

Restriction endonuclease digestion and agarose gel electrophoresis- Purified MAC genomic DNA was used for restriction digestion with EcoRI and BamHI. Digested DNA was electrophoresed on 0.7% and 1.5% agarose gel, stained in ethidium bromide and visualised over a Pdi gel documentation system (8a).

Construction of recombinant plasmids - Plasmid vector PBR 322 was harvested and purified using a standard technique (8b). The purity and DNA concentration was checked as per recommended procedure (8c). The method described by Maniatis *et al* was followed for ligation and transformation of the competent *E. coli* DH5 α cells (8d).

Screening of recombinant DNA clones - After transformation, colonies were grown on Luria Bertani Agar (LBA) and transferred to LBA-ampicillin plate. Colonies that grew on LBA-ampicillin were subcultured on LBA-tetracycline plate. The colonies that failed to grow on LBA-tetracycline were picked up as recombinant plasmid clones (8e).

Purification of cloned fragment and preparation of labeled probe - The cloned MAC genomic DNA fragment was recovered from the recombinant plasmid by digestion with EcoRI, subsequent electrophoresis and electroelution (8f).

The purified DNA 0.1 to 0.2 μ g and 10 X bacteriophage T₄ DNA polymerase buffer, 2 μ l, were mixed and volume made to 19 μ l with sterile water. A solution containing three of four dNTPs, each having a concentration of 2 mM, 2 to 10 μ Ci of an aqueous solution of dATP (labeled with P³², specific activity 400–3000 Ci per m mole) and bacteriophage T₄ DNA polymerase 1 μ l (2.5 units) was added to the DNA solution. The reaction mixture was incubated at 37°C for five minutes and 1 μ l of 2 mM solution of the unlabeled dATPs was added. The reaction was stopped by heating at 70°C for five minutes and the labeled DNA was separated by centrifugation through small column of Sephadex G-50 (9).

Hybridization procedure- Nineteen strains of MAC isolated from biological samples were digested with EcoRI. Digestion was continued for 4 hours and the reaction was terminated by adding the tracking dye. Digested and separated DNA bands were visualised under UV transilluminator. Gel was passed in solutions of 0.25 M HCl, 1.5 M NaCl, 0.5 N NaOH, 1M Tris/1.5M NaCl to denature the DNA. Digested MAC genomic DNA was transferred from gel to solid support on Hibond nylon (SIGMA) membrane according to the Southern blot capillary transfer method and hybridization with P³² labeled DNA probe was done at 68°C in presence of 2x saline-sodium citrate (SSC) buffer (9).

RESULTS

MAC isolate 479/93, demonstrating biochemical characters of a typical MAC strain was used for preparation of genomic DNA for cloning. The concentration of purified MAC genomic DNA was found to be 0.29 μ g/ml (yield 0.4 mg/gm cell pellet).

The quality of mycobacterial DNA was determined by OD₂₆₀/OD₂₈₀ ratio. The ratio was 1.6 for the DNA preparation used in this study. The purified genomic DNA was digested with BamHI and EcoRI. Digestion with BamHI or EcoRI resulted in appearance of DNA as smears on agarose gel electrophoresis.

For isolated PBR 322, OD₂₆₀/OD₂₈₀ ratio was 1.4. Plasmid vector PBR 322 showed as two bands on agarose gel electrophoresis but after complete digestion with BamHI or EcoRI, only one band was seen.

Mycobacterial DNA alone or linearised dephosphorylated PBR322 alone did not cause transformation of competent *E. coli* cells. Colonies growing on LBA - ampicillin but not on LBA - tetracycline were identified (fig.1) and subcultured for further studies.

Figure1. Screening of transformants for recombinant plasmids. Colonies grown on LBA-ampicillin plate (left) were subcultured on LBA- tetracycline plate (right). Colonies that fail to grow on LBA-tetracycline have recombinant plasmid (↑).

Genomic MAC DNA of nineteen strains was digested with *EcoRI*, run in 0.7% agarose gel and then transferred to nylon membrane by Southern blotting. A 16 kb cloned radiolabeled DNA fragment was used as a probe. Genomic DNA of *Mycobacterium tuberculosis* (three strains, two of these three strains are shown in Lanes 2 and 3 of fig. 2) and *Mycobacterium kansasii* (one strain, Lane 6 of fig. 2) were used as genus level control and were subjected to same processing as for MAC. Twelve MAC strains and two *M. tuberculosis* strains demonstrated hybridization with labeled, cloned DNA probe. Seven MAC strains and two controls (one strain each of *M. tuberculosis* and *M. kansasii*) showed no hybridization.

Figure 2. MAC genomic DNA purified from clinical isolates (digested with *EcoRI*) and probed with a cloned 16 kb probe (P^{32} labeled)

Control (C) 1: *M. tuberculosis* (clinical isolate)- Lane 2; 2: *M. tuberculosis* (H37Rv) –Lane 3; 3: *M. kansasii* –Lane 6

Test : Lanes 1,4,5,7-12. No band seen in Lanes 8 and 9. Other test strains showed single or multiple bands.

Marker(M): λ DNA double digested with *Hind III* and *EcoRI*.

Figure 3. MAC genomic DNA purified from clinical isolates (digested with *EcoRI*)and probed with a cloned 16 kb probe (P^{32} labeled). Lane 17 shows strain 479/93 . No band seen in Lanes 13,14 and 16. Other test strain showed single or multiple bands.

DISCUSSION

A range of physical and chemical methods have been described to lyse the tough mycobacterial cell-wall (7,10-18). The chemical methods vary in their effectiveness and good physical methods have not yet been developed that are without risk of infection to laboratory personnel or shearing of genomic DNA. Bose *et al*, 1993 reported a rapid and gentle method for preparation of quantitatively and qualitatively superior mycobacterial DNA (7). Prior treatment of mycobacterial cells with cesium chloride greatly facilitated the susceptibility of these cells to further digestion and extraction procedures. The mycobacterial DNA was fully digestible by both BamHI and EcoRI and was used for cloning into the plasmid vector PBR 322. BamHI cuts the tetracycline-resistance gene of PBR 322. Formation of recombinant plasmid using restriction enzyme BamHI causes inactivation of tetracycline-resistance gene making the screening of recombinant plasmid easier (8).

Roberts *et al*, 1987 used whole chromosomal DNA probes to identify isolates of *M. tuberculosis*, MAC and *M. gordonae*. The probe for *M. tuberculosis* was prepared from *M. bovis* BCG and for MAC from the three strains representing each of the three DNA homology groups in MAC. The dot blot identified 57 of the 61 (93%) cultures grown on the solid media and 100% of antibiotic-treated broth-grown cells were correctly identified. When the probes were tested against 63 positive BACTEC cultures of clinical specimens, 59% were correctly identified (2). Cousins *et al*, 1996 used multiplex PCR

to correctly identify 33 of 34 isolates identified as *M. avium* and all 51 isolates as *M. intracellulare* (5). Pestel-Caron and Arbeitt, 1998 used Pvu II to use IS 1245 polymorphisms among 159 *M. avium* isolates that represented 40 distinct *M. avium* strains. Although Southern blots probed with a fragment of IS 1245 typically demonstrated strongly hybridizing fragments, bands with a disproportionately weaker signal were also observed for some isolates. Such fragments were characteristic of low-copy number patterns and were observed even under stringent hybridization conditions (19).

In the present study, twelve MAC strains out of nineteen showed positive hybridization although a few showed very faint bands (data not shown). While the faint banding could be because of poor initial restriction digestion or insufficient transfer on Southern blotting, the possibility of wide ranging strain variation cannot be ruled out. The same reasonings can be applied for no hybridization in case of seven strains. Positive hybridization was noted in two *M. tuberculosis* strains which could be due to shared DNA homology. No signal was received from *M. kansasii* however. As the cloned DNA fragment used to make the probe was large, some bands may have been obtained due to partial hybridization with test DNA. Some of the bands may have been missed because of the partial denaturation or self annealing of the probe. Further work is on to screen the genomic DNA library to obtain a clone that would be specific for MAC with no cross-reaction with *M. tuberculosis*.

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