GENOTYPIC DETECTION OF MUTATIONS IN RIFAMPICIN RESISTANT CLINICAL ISOLATES OF *MYCOBACTERIUM TUBERCULOSIS*

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ABSTRACT

The resistance of majority of *M. tuberculosis* strains to rifampicin a key drug in the tuberculosis therapy, has been cause of major alarm world-wide. Such an association of strain with the drug takes a long time i.e. 2-3 months. We have modified a method for rapid detection of rifampicin resistant strains by screening them through PCR-SSCP.

We have identified mutations in *rpo*, β region of the gene encoding, β sub-unit of RNA polymerase in rifampicin resistant 67 clinical isolates of *M. tuberculosis*. Mutations were screened in these isolates by single strand confirmation polymorphism (SSCP). When DNA sequencing data was compared with the result of SSCP analysis, direct PCR sequencing results were more easily interpreted and contained more sequence dependent information. These findings provide the basis for rapid detection of rifampicin resistance, a marker of multidrug resistance.

KEYWORDS : Genotypic detection, Rifampicin resistance, mutation, *Mycobacterium tuberculosis*.

INTRODUCTION

The resistance of *Mycobacterium tuberculosis* strains, particularly the ones isolated from cases of HIV, to antituberculosis drugs has led to an overall interest in studying molecular basis of drug resistance.

The resistance of majority of *M. tuberculosis* strains to rifampicin, a key drug in tuberculosis therapy, has been a cause of major alarm world wide (1, 2, 3). Recently, the molecular basis of rifampicin-resistance in *M. tuberculosis* was identified by Telenti et al (4). and Kapur et (5). They reported distinct mutations present in the gene, *rpo* β , encoding β Subunit of RNA polymerase.

In this study we describe the genotypic detection of mutation in rifampicin resistant (6, 7) clinical isolates of *M. tuberculosis* in *rpo* β region of gene by SSCP, direct PCR sequencing and also by automated DNA sequencing.

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MATERIALS AND METHODS

SAMPLE : 67 clinical isolates were collected from Lala Ram Sarup Institute of tuberculosis and allied diseases, New Delhi. These isolates were sub-cultured in Middlebrook's 7H11 medium and their susceptibility to rifampicin was determined by radioactive method (Bactec System) (6).

DNA : DNA of *Mycobacterium tuberculosis* was isolated by the published procedure (7).

PCR : PCR amplification was performed based on the method of Kapur et al (5). A 350 base pair fragment of *rpo* β gene was amplified using the following oligonucleotide primers.

forward 5'-GGGAGGCGGATGACCACCCA-3'

reverse 5'-GCGGTACGGCGTTTCGATGAAC-3'

The following thermocycle parameters were used : 30 cycles of denaturation at 94°C for 1 min, primer annealing at 56°C for 1 min, extension at 72°C for 4 min.

Composite gel (acrylamide and agarose)

Composite gel was prepared with 5% acrylamide and 0.25% agarose. 30% acrylamide 29 : 1 (Sigma Chemical Co., USA) in 20 × TBE buffer (Tris borate 180 mM, 4 mM, EDTA, PH 8.0). The temperature of acrylamide and agarose was brought to 27°C in a water bath before the two solutions were mixed. After the addition of 200 µl 10% ammonium sulphate and 15 µl tetramethylethylenediamine, the mixture was immediately poured into vertical gel apparatus. The gel was allowed to set for 1 hr at room temperature.

25 ml of composite gel was prepared for 200 × 220 × 0.75 mm gel plate.

Single strand confirmation polymorphism (SSCP)

20 µl of PCR product was mixed with equal volume of 95% formamide containing 0.5% xylene cyanol, 10 mM NaOH, 20 mM EDTA and 0.5% bromophenol blue. The sample was boiled for 5 min at 95°C. Electrophoresis in composite gel takes 2-3 h.

Sequencing of PCR Product

PCR product was purified by microcon 100 (Amicon, Inc; Beverly, Mass). Sequencing reaction was performed by Promega kit. DNA sequencing of *rpo β* region of gene was also analysed by automated DNA sequencing.

RESULTS AND DISCUSSION

Presently, detection of *M. tuberculosis* strains and multidrug resistance (MDR), particularly resistance to rifampicin and isoniazid requires a very long time ranging from 2-3 months. Meanwhile patients are prescribed empirically and they remain infectious. Our description of the molecular basis of resistance to rifampicin in *M. tuberculosis* have important implications for the development and modification of new strategies for

rapid detection of drug resistant strains of *M. tuberculosis*.

Rifampicin binds to RNA polymerase of β subunit in a locus formed by the appropriate complexing of the different RNA polymerase subunits (7). If there was any change in substitution of key aminoacids it would result in defective binding of the drug. We have used PCR-SSCP technique for rapid screening of mutations by the methodology (8) as modified by Khanna et al. (9). Figure 1 shows that the mobility shifted in resistant samples, hence this technique is promising for identification of rifampicin resistant clinical isolates. PCR-SSCP allows processing of large number of samples, with results available within 48 h.

Fig. 1 : Mutations identified by PCR-SSCP analysis of the amplified fragment of *rpoβ* gene of *M. tuberculosis*.

R = rifampicin resistant, S = sensitive

Our sequencing data indicated that most of the mutation were on 69-bp segment. Out of 67 samples, 3 samples were analysed by automated DNA sequencing and 10 samples were used for direct PCR. Point mutations were observed in a total of 15 samples. These isolates were resistant to rifampicin and were confirmed to be resistant to this drug by conventional methods.

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