Rapid detection of mutations in \textit{rpoB} gene of rifampicin resistant \textit{Mycobacterium tuberculosis} strains by line probe assay

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Received January 10, 2003

\textbf{Background & objectives:} Multidrug resistant (MDR) tuberculosis (TB) is a problem of increasing importance in the world due to limited treatment options. Resistance to rifampicin results from nucleotide changes in the gene encoding the \(\beta\) subunit of the RNA polymerase (\textit{rpoB}) of \textit{Mycobacterium tuberculosis}. Rifampicin resistance is considered as a marker for MDR TB. The nature and frequency of mutations in the \textit{rpoB} gene of rifampicin resistant clinical isolates vary considerably according to the geographical location and very little information is available on specific mutational patterns in India. This study was undertaken to detect and characterize the \textit{rpoB} gene mutation associated with rifampicin resistance in \textit{M. tuberculosis} by line probe assay.

\textbf{Methods:} A total of 36 strains of \textit{M. tuberculosis} were analysed by INNO-LiPA Rif TB and compared with the results of conventional susceptibility testing method. After PCR amplification of the region of RNA polymerase involved in rifampicin resistance, the amplified product was hybridized with a set of 10 oligonucleotides immobilized onto a membrane strip. From the pattern obtained, the presence or absence of rifampicin resistance in the \textit{M. tuberculosis} strains was assessed.

\textbf{Results:} It was found that the \textit{M. tuberculosis} probe was 100 per cent specific; the most frequently observed mutation was His-526-Tyr in the \textit{rpoB} gene; and correlation between the results of the LiPA and those obtained by the classical susceptibility testing was excellent (100%).

\textbf{Interpretation & conclusion:} INNO LiPA was found to be a reliable, simple, rapid and informative tool for the early detection and characterization of \textit{rpoB} mutation associated with rifampicin resistance in \textit{M. tuberculosis} in the clinical laboratory setting and may constitute an important molecular method for the control of tuberculosis.

\textbf{Key words} INNO LiPA Rif TB - \textit{Mycobacterium tuberculosis} - reverse hybridization - rifampicin resistance - \textit{rpoB} gene

Multidrug resistant (MDR) tuberculosis, \textit{i.e.}, resistance to at least rifampicin (RMP) and isoniazid (INH), is an increasing problem both in the developed as well as in the developing countries\textsuperscript{1}. The problem is even more serious among the human immunodeficiency virus (HIV) infected population\textsuperscript{2-4}. The early diagnosis of tuberculosis and the rapid detection of resistance to the major anti-tubercular drugs is therefore, of utmost importance for the effective control of the resurgent epidemic. However, convenient methods for the rapid detection of drug resistance in \textit{Mycobacterium tuberculosis} are still not available. Routine anti-mycobacterial susceptibility testing still depends on culture methods. But, primary culture can take up to 6 wk before a definitive result can be obtained and another 2-4 wk are required for drug susceptibility testing. Recently, the genetic basis of the resistance against the anti-tubercular drugs has been unraveled\textsuperscript{5,6}. Rifampicin resistance is most commonly due to point mutations and small insertions and deletions in the \textit{rpoB} gene, which codes for the \(\beta\)
subunit of RNA polymerase in *M. tuberculosis*. This drug is a key element in the treatment of tuberculosis and is also a useful surrogate marker for MDR tuberculosis\(^7,8\). The nature and frequency of mutations in the *rpoB* gene of rifampicin resistant clinical isolates of *M. tuberculosis* vary considerably according to the geographical location\(^9\). The genetic basis for RMP resistance in approximately 95 per cent of the cases is due to mutations in an 81-bp rifampicin resistance determining region (RRDR) of the *rpoB* gene corresponding to 507-533 codons that codes for the β subunit of RNA polymerase of *M. tuberculosis*\(^10\). However, there is very scanty information available on the specific mutation pattern and its detection in the *M. tuberculosis* strains isolated from our country. In the present study, mutations in the *rpoB* gene of rifampicin resistant isolates, mostly from the northern part of India, were analyzed and its rapid detection by reverse hybridization based line probe assay was evaluated.

**Material & Methods**

A total of 200 strains of *M. tuberculosis* isolated from sputum samples of patients of tuberculosis during February to July 2002, were used for this study. The confirmation and identification of strains of *M. tuberculosis* complex were done by standard conventional culture and biochemical tests\(^11\). Drug susceptibility tests were performed on these strains by standard proportion method\(^12\).

Of the 200 strains, thirty rifampicin resistant strains (40 µg rifampicin/ml was used as the critical concentration) and 6 rifampicin sensitive strains (as determined by the proportion method) were then evaluated by the line probe assay for any specific mutational pattern of *rpoB* gene. One strain each of *M. tuberculosis* \(H_37R_v\) (obtained from Central JALMA Institute for Leprosy, Agra, India) and *M. flavescence* (clinical isolate) were used as the controls.

**Sample preparation for DNA study:** A loopful of bacteria was suspended in 500 µl of TE buffer (10 nM Tris, 1 mM EDTA, pH 8) and the suspension was incubated for 10 min at 95°C. After centrifugation (13000 g for 5 min), 1 - 10 µl of the supernatant was used for the PCR, which was performed in a final volume of 50 µl containing 50mM KCl, 10 mM Tris-HCl (pH 8.3), 2.2 mM MgCl\(_2\), 200 µm (each) deoxynucleoside triphosphate, and 0.01 per cent gelatin and 1 U of AmpliTaq polymerase (Perkin-Elmer, USA). The primers (provided with the kit) used were IP1 5’ GGTCGGCATGTCGCGGATGG 3’ biotinylated at 5’ and IP2 5’ GCACGTCGCGGACCTCCAGC 3’ biotinylated at 5’, each used at a concentration of 20 pmol per reaction mixture. The primers amplified a 256 bp region of the *rpoB* gene. The following cycling protocol was applied: initial denaturation at 94°C for 3 min and 35 cycles of 45 sec at 94°C, 1 min at 64°C and 45 sec at 72°C, followed by a final cycle of 10 min at 72°C.

**Line probe assay (LiPA):** The line probe assay (INNO-LiPA Rif TB; Innogenetics, Belgium) is based on the reverse hybridization principle\(^13\). Specific oligonucleotides are immobilized at known locations on membrane strips and are hybridized under strictly controlled conditions with the biotin-labeled PCR product. Five partially overlapping wild type probes (S1 through S5) of 19 to 23 bases were designed. These S probes overlap the entire *rpoB* region of relevance and exclusively hybridize to the wild-type sequence. If a mutation is present in one of the five regions, the corresponding probe will be prevented from hybridizing under the stringent hybridization and washing conditions used. Consequently, the absence of hybridization signal with one of the probes would be indicative of the presence of mutation, implying resistant genotype. Hence this set of probes suffices to detect a mutation but does not give precise information about the mutation. For this, four mutant (R) probes (19 or 23 bases) were designed. These R probes, R2, R4a, R4b, and R5, hybridize with sequences of the four most commonly observed mutations *viz.*, Asp-516-Val, His-526-Tyr, His-526-Asp, and Ser-531-
Leu, respectively. The assay was performed according to the manufacturer’s instruction. Key to interpretation of the results of INNO LiPA strips is shown in the Table.

**Results & Discussion**

Detection of mutations in the *rpoB* gene of *M. tuberculosis* has been reported to be an accurate predictor of rifampicin resistance\(^8\). It is, however, usually associated with isoniazid resistance and as such rifampicin resistance may be a potential marker of MDR in *M. tuberculosis*\(^7,8\). The PCR based line probe assay used in this study is able to detect and identify the mutational pattern in the *rpoB* gene of *M. tuberculosis* and simultaneously provide information on the susceptibility of a strain to rifampicin.

<table>
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<tr>
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According to the reactivities of the probes, 10 regular LiPA patterns can be expected for strains of the *M. tuberculosis* complex. If all R probes are negative and all S probes are positive, a wild type sequence is present. When one of the S probes is missing and no R probe hybridizes, this pattern is described with a Δ preceding the missing probe (*e.g.*, ΔS1). If one of the S probes is negative and the corresponding R probe is positive, the pattern is described according to the R probe observed (*e.g.*, R2).
Representative INNO LiPA results for the rifampicin resistant strains of *M. tuberculosis*. The first three strips represent the 28 strains that did not hybridize with S4 probe but showed the presence of R4a probe showing the mutation His - 526-Tyr. The next two strips show the 2 strains that showed absence of the S3 probe but did not show any mutant or R probe. The exact area of mutation thus could not be determined in these two. The sixth strip shows the pattern obtained from hybridization with *M. flavescence*, which was kept as a control. The *M. tuberculosis* complex specific probe is absent in the strip.

For all *M. tuberculosis* strains studied (n=36), the set of primers used generated an amplified product of expected length (approximately 256 bp). A probe specific for *M. tuberculosis* complex nucleotide sequence included on each strip of the line probe kit was clearly positive for each of the 36 strains and H37Rv but negative for *M. flavescence* (Fig.). It was found that all the 30 strains (which were rifampicin resistant by the proportion method) showed two types of mutational pattern in the LiPA strips.

In 28 of the 30 samples (93.3%), one of the wild (S) probes (S4), failed to hybridize, and a positive hybridization signal with R4A indicated the presence of His-526-Tyr mutation. In the other 2 (6.66%), the precise mutation could not be localized. Both of these did not hybridize with the S3 probe and none gave a positive result with the mutant (R) probes (Fig.). World-wide, the most frequent mutations are Ser-531-Leu, followed by His-526-Tyr and His-526-Asp. The majority of the mutations occur in the region from codons 526 to 533 bp. DNA sequencing of the *M. tuberculosis* strains could not be done in our study but excellent concordance (94-100%) of automated sequencing results and INNO LiPA Rif TB results have been reported. In only two strains (6.66%), the precise mutation could not be localized by LiPA. This could be due to the fact that the mutations might be in areas for which there is no mutant probe incorporated in the LiPA strips. This highlights the importance of developing systems based on mutations in indigenous strains. Novel mutations in Indian isolates have already been identified. *In vitro* sensitive strains were always correctly identified by LiPA. Thus excellent correlation was found between LiPA and the proportion method (100%). This is in agreement with other studies, which have shown excellent concordance between the line probe assay and phenotypic susceptibility tests.
Thus the INNO LiPA Rif TB proved to be a simple, rapid and reliable tool both for the identification of \textit{M. tuberculosis} and for the characterization of the \textit{rpoB} gene mutations responsible for the resistance to rifampicin before the results of the conventional method are available. Moreover this assay has also been used to detect the presence of \textit{M. tuberculosis} complex and its resistance to rifampicin directly from clinical samples and needs further evaluation.

References


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