**Correlation of mutations detected by INNO-LiPA with levels of rifampicin resistance in *Mycobacterium tuberculosis***


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*Background & objectives*: Due to emergence of drug resistance in *Mycobacterium tuberculosis*, there is a need to have accurate and rapid methods for detection of drug resistance to important drugs like rifampicin. The present study was aimed at evaluation of a commercially available INNO-LiPA assay, for the detection of mutation in *rpoB* gene region of *M. tuberculosis* and correlate these mutations with levels of rifampicin resistance for assessing their clinical relevance.

*Methods*: Fifty five well-characterized isolates of *M. tuberculosis* deposited from various regions of India in Mycobacterial Repository Centre at the CJILOMD, Agra were subjected to susceptibility testing for rifampicin at various concentrations of drug viz., 10, 40, 64, 128 µg/ml on Lowenstein-Jensen (LJ) medium. *rpoB* gene fragment (260 bp) was amplified using Rif-TB amplification kit and after hybridization, detection was done by using INNO-LiPA Rif TB kit.

*Results*: The *rpoB* gene could be amplified from DNA extracted from all the 55 culture isolates and showed clear hybridization pattern with *M. tuberculosis* complex specific probes on LiPA strips. Mutations detected were correlated with degree of rifampicin resistance. All the sensitive isolates (identified by MIC) were identified as rifampicin sensitive (100%) by INNO-LiPA as they exhibit positive for wild type ‘S’ probes and negative for ‘R’ probes. Two of the 5 isolates, resistant at 10 µg/ml and 40 µg/ml had either D516V, H526Y mutations or unknown mutations. Thirty (85.71%) isolates resistant at clinically relevant levels (64,128µg/ml) exhibited double, triple or more ‘R’ type mutations (R2(D516V), R4a(H526Y), R4b(H526D), R5(S531L)) as well as unknown mutations present at ‘S’ probes region whereas remaining isolates did not show any mutation by this method. This method could identify with definitiveness 60 per cent (21/35) isolates as rifampicin resistant as mutations observed in others were also present in isolates with low levels of resistance.

*Interpretation & conclusion*: The results indicate that INNO-LiPA Rif TB test is a rapid and easy to use method for detection of mutations associated with rifampicin resistance in *M. tuberculosis*. However, as some of these mutations are also present in isolates with low degree of resistance which are still microbiologically sensitive to rifampicin, there is a need to improve this assay by exclusion of some of the current probes and inclusion of more probes.

**Key words** *M. tuberculosis* - mutation detection - *rpoB* gene analysis - sensitivity
overall trends show that acquired resistance is more common than primary resistance. In India, the overall figure for MDR is very low and varies from 0 to 6 per cent. Primary MDR is considered to be less than 4 per cent. However, acquired MDR has ranged from < 6.0 to 18.5 per cent in different reports.

As rifampicin is the most important drug for treatment of tuberculosis, it is important to rapidly detect the resistance to rifampicin. Since microbiological assays are time consuming, tremendous interest in the development and use of molecular methods has been generated. Mutations in rpoB gene region have been shown to be associated with resistance to rifampicin in M. tuberculosis. INNO-LiPA test developed by Beenhouwer et al. based on reverse blot hybridization of different regions of rpoB gene has been reported to be useful in identification of such mutations. This is a rapid system and holds promise in clinical practice. As the type of strains and mutations may vary from country to country and in various regions of a country, actual experience of application of this system is necessary. The present study was undertaken to evaluate a commercially available assay for the detection of mutations in rpoB gene of M. tuberculosis isolates and assess the correlation of such mutations with levels of resistance to rifampicin.

Material & Methods

Samples: Biochemically confirmed culture isolates of M. tuberculosis (n=55) collected from patients from different regions of India (Ranchi, Manipal, New Delhi, Chandigarh, Agra, Bangalore, Ahmedabad, Cochin and Jammu & Kashmir) deposited in Mycobacterial Repository Centre, at Central JALMA Institute for Leprosy and other Mycobacterial Diseases (CJILOMD), Agra, were included in this study.

Subculture: The isolates were recultured on Lowenstein Jensen (LJ) medium [Ingredients: Hen's egg, magnesium citrate (BDH, England), magnesium sulphate (Glaxo, Mumbai), KH₂PO₄ (Glendia Ltd., New Delhi), malachite green (Hi-Media, Mumbai), L-asparagine (Hi-Media, Mumbai), glycerol (S.D. Fine Chemicals, New Delhi)] and incubated at 37°C until growth obtained or up to a maximum of 8 wk.

Susceptibility testing: M. tuberculosis isolates included in the study were subjected to susceptibility testing by minimum inhibitory concentration (MIC) method at different concentrations of the drug. A standard bacterial suspension (wet weight 4 mg/ml) was inoculated on LJ medium with a loop of 3 mm internal diameter. For sensitivity, rifampicin (Sigma, USA and Novartis, India Ltd.) was incorporated in LJ medium to give final pre-inspissations concentrations of 10, 40, 64 and 128 µg/ml. MICs were determined by using standard criteria of counting the colony forming units (cfu) and comparing with the culture. Both plain and drug containing media were incubated at 37°C and results were read after 28 days.

Molecular studies

DNA extraction: DNA from each isolate was extracted by the procedure of van Embden et al. using lysozyme/proteinase-k (Bangalore genei), deproteinization and precipitation with isopropanol (Ranbaxy, India).

Polymerase chain reaction (PCR): Amplification of the regions of the gene encoding for rifampicin resistance for the β subunit of RNA polymerase (rpoB) was done in a thermal cycler (model PT-100, MJ Research, USA) using Rif-TB-Amplification kit (INNO-LiPA-Innogenetics N.V., Belgium) according to the manufacturer's instructions. rpoB gene fragment (260 bp) was amplified by using specific biotinylated primers (complementary to conserved regions flanking the target sequence). Briefly, Rif-TB-amplification profile was denaturation at 95°C for 1 min, annealing of primers at 55°C for 1 min and extension of primers at 72°C for 1 min. Thus biotinylated exact copy of the template-sequence was produced after one cycle of denaturation, annealing and extension. A total of 30 cycles were carried out.

Line probe assay: After gel analysis, amplified biotinylated DNA was denatured and hybridized with specific oligonucleotide probes immobilized as parallel lines on membrane-based strips provided with line probe assay kit (INNO-LiPA-Rif-TB, Innogenetics N.V., Belgium), followed by a colour detection step as per manufacturer's instruction. In this assay wild type probes (S₁-S₅) overlap the entire relevant rpoB gene region and hybridize specifically to the wild type sequences.
(S1-L511P, L511R, S512T, Q513K, S512T, D516Y, D-516E, D516G, S1-L511P, S512T, Q513K, S2-D516Y, D516E, D516G, 517-518, S3-S522L, S4-H526N, H526Q, H526R, H526L, H526T, H526C, S5-S531C, L533P codon). This set of probes is able to detect a mutation but does not provide specific information concerning the mutation present. Four additional mutant probes (R) are specifically designed to hybridize the sequences of the four mutations most frequently observed (R1 mutation-D516V, R4a mutation-H526Y, R4b mutation-H526D, R5 mutation-S531L). If a mutation is present in one of the probe target regions, the mismatch created will present the corresponding probe from hybridization (∆, no signal obtained). The sensitive strains give positive signals with wild type probe (S) and for resistant type at least one of the mutant probe (R) gives positive signal.

Results

Sensitivity levels to rifampicin among 55 isolates of M. tuberculosis were assessed by the MIC method and isolates were categorized into two major groups: Microbiologically Rif sensitive Gp I and definite resistant Gp II. Microbiologically Rif sensitive Gp I consisted of three subgroups - complete sensitive sub gp i, intermediary sub gp ii and intermediary sub gp iii. Sub gp i had 10 isolates sensitive at all levels i.e., 10 µg/ml or more of rifampicin. Sub gp ii had 5 isolates resistant at 10 µg/ml and sensitive at 40, and 64 µg/ml, and sub gp iii also had 5 isolates resistant at 10, 40 µg/ml and sensitive at 64 and 128 µg/ml. Definite resistant Gp II had 35 isolates resistant at 10, 40, 64 and 128 µg/ml of rifampicin (Table I). By this line probe assay all 10 isolates from sub gp i were identified as rifampicin sensitive as they exhibited wild type pattern, S probes (S1, S2, S3, S4, and S5 - unknown mutations) were positive and hybridization was negative with all the 4 mutant probes, R probes (R2, R4a, R4b, R5) were negative. Thus in this complete sensitive sub gp i, 100 per cent concordance with sensitivity was obtained (Table I). In intermediary sub gp ii (resistant at 10 µg/ml only) one of the 5 isolates showed mutation at R2(D516V) and R4a(D526Y). Similarly in intermediary sub gp iii (resistant at and up to 40 µg/ml only) 2 of the 5 isolates showed mutation at R2(D516V) as well as R4a(D526Y), whereas in these groups probe did not bind to mutation R4b(H526D) and R5(S531L). Three of the 5 isolates of sub gp ii and 2 of 5 sub gp iii isolates failed to hybridize to ∆S, ∆S probes and one each sub gp ii and iii isolate hybridized with wild type ‘S’ probes (unknown mutations) but did not hybridize with ‘R’ probes. Thus in these intermediary sub gp ii and iii 70 per cent concordance was found (Table II). In the definite resistant Gp II of isolates exhibiting high degree of resistance (MIC 64 µg/ml or above) mutations were present in 30 of 35 isolates (85.71%). Among these 30 isolates, 9 had single mutation (5 had R2(D516V), 1 had R4a(H526Y) and 3 had R5(S531L)) and 17 had two or more mutations (1 had R4a(H526Y) and R4b(H526D), 7 had R2(D516V), R4a(H526Y), R4b(H526D) and R5(S531L)) and 4 had R2(D516V), R4a(H526Y), R4b(H526D), R5(S531L) common

| Table II. Concordance between INNO-LiPA assay and rifampicin sensitivity profile of M. tuberculosis |
|---|---|---|
| Sensitivity profile Groups | Mutation detection by INNO-LiPA | INNO-LiPA concordance with rifampicin sensitivity profile |
| Microbiologically Rif sensitive Gp I | | |
| (i) Complete sensitive sub gp i | 0/10 | 10/10 (100%) |
| (ii) Intermediary (Rif 10, 40 µg/ml) sub gp ii, iii | 3*/10 | 7/10 (70%) |
| Definite Rif resistant Gp II | 30*/35 | 30* / 35 (85.71 %) |

*In 9 of these isolates some mutations were common with isolates with low degree of resistance (Microbiologically sensitive); Gp, group
with low degree of resistance whereas 4 of these 35 isolates did not hybridize with S(L511P), S(533) probes. Five isolates hybridized with ‘S’ probe and did not show positive signals with any of the mutant probe (R) in ‘definite resistant Gp II isolates.

Discussion

Accumulation of genomic mutations in individual drug target gene is considered as the primary mechanism of MDR-TB. Rifampicin is often regarded as an excellent surrogate marker for MDR-TB. As mutations in rpoB gene are generally considered to be associated with resistance in most of the isolates of M. tuberculosis, INNO-LiPA assay was developed by Beenhouwer et al7. This assay has been found to be generally satisfactory and directly applicable to isolates of M. tuberculosis as well as clinical samples7.

In the present study the probe specific for M. tuberculosis complex nucleotide sequences’ included on each strip of line-probe kit was positive for 55 culture isolates included in the study which were confirmed as M. tuberculosis by biochemical tests10. This technique did not find any mutations in the 10 sensitive isolates. Different mutations such as type R2 (D516V) , R4a (H526Y) and R5 (S531L) were found in isolates singly or in combination. Deletion of wild type Δ S probes (Δ S1,4,5) were also present showing some unknown resistance in most of the isolates of gene are generally considered to be associated with surrogate marker for MDR-TB. As mutations in rpoB gene is considered as the primary mechanism of ‘definite resistant Gp II isolates.

In conclusion, INNO-LiPA strips showed applicability of the assay and concordance of results of isolates to detect resistance in few hours, but four ‘R’ type mutations were not sufficient to detect resistance in all the resistant isolates. As we are missing the targets, there is a need to add more probes based on novel mutations reported by different investigators8,10,11,12,13 to develop effective tool for more efficient detection of rifampicin resistance for M. tuberculosis, so that this technology could be made applicable to patient care in India as well as elsewhere.

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References


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