

Rapid Genotypic Detection of *rpoB* and *katG* Gene Mutations in *Mycobacterium tuberculosis* Clinical Isolates from Northern India as Determined by MAS-PCR

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Background: There is a growing need to develop rapid laboratory research methods to counter the menace of drug resistant tuberculosis (MDR-TB) cases worldwide especially in developing countries. The present study was undertaken to investigate the type and frequency of *rpoB* and *katG* mutations in rifampicin (RIF) and isoniazid (INH) resistant strains respectively of *Mycobacterium tuberculosis* (MTB) circulating in Northern India and to explore the utility of multiplex-allele-specific (MAS)-PCR assay for detection of drug-resistant MTB isolates in low resource set up. **Methods:** Phenotypic and genotypic drug susceptibility testing (DST) was performed on 354 MTB isolates. **Results:** Mutation in *rpoB* gene was found

most frequently at codons 531, 526 and 516 (59.83%, 45.29% and 22.22%, respectively). Further, combinations of 2–3 point mutations were also observed in 19.66% of RIF-resistant MTB strains. The frequency of mutations in *katG* gene was found at codon 315 among 82.95% of the INH-resistant MTB isolates. MAS-PCR detected *rpoB* and *katG* mutations in phenotypically resistant isolates with sensitivities of 93% and 83% respectively. **Conclusion:** MAS-PCR assays can be used for rapid detection of drug-resistant TB strains in routine diagnostic practice, enabling early administration of appropriate treatment regimens to the affected patients. J. Clin. Lab. Anal. 27:31–37, 2013. © 2012 Wiley Periodicals, Inc.

Key words: Drug-resistant TB; rifampicin; isoniazid; point mutation; sensitivity

INTRODUCTION

India has the highest number of tuberculosis (TB) cases in the world with the situation worsening by the appearance of drug resistant especially multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains of *Mycobacterium tuberculosis* (MTB). A large scale population based survey in several states of India indicated multidrug resistance levels of 3% among new TB cases and 12–17% among previously treated TB patients. Further, the annual incidence of MDR-TB is estimated to be 99,000 cases (1). MDR-TB is a form of TB that is difficult and expensive to treat because it fails to respond to two important first-line drugs rifampicin (RIF) and isoniazid (INH) (2). The development of drug resistance may be a tragedy not only for the patients himself but for others as they can infect other people with their drug-resistant organisms. To meet the targets set in the global plan, diagnosis and treatment of MDR-TB needs to be rapidly scaled up in five highly affected countries including India (3).

RIF together with INH is the most important cocktail of drugs used to treat TB. Many studies have reported that mutations in three codons (516, 526, or 531) of the *rpoB* gene explained the majority (70–95%) of RIF resistant strains (4). Further, approximately 50 to 95% of INH-resistant strains have been found to contain mutations in codon 315 of the *katG* gene (5, 6).

Presently, only 2% of MDR-TB cases worldwide are being diagnosed, mainly because of inadequate laboratory services (7). Conventional drug-susceptibility testing (DST), still considered as gold standard method for

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determination of drug resistance, has limitations of long turnaround time of about 4–6 weeks. Modern broth-based culture systems, such as Mycobacteria Growth Indicator Tube MGIT 960 (Becton Dickinson, Cockeysville, MD) or BacT/ALERT 3D (bioMerieux, Durham, NC), are somewhat quicker but are expensive and need sophisticated equipments. To address this growing crisis, WHO is repeatedly suggesting that developing countries have to use rapid molecular-based assays for the diagnosis of drug-resistant TB bacilli (8, 9). Molecular tests can provide results within a day or two and thus enable us for early and appropriate treatment of the drug-resistant cases. This not only reduces morbidity and mortality of MDR TB cases but also interrupt transmission of drug-resistant TB strains.

Multiplex allele-specific PCR (MAS-PCR) assay was first described by Mokrousov et al. (10) for the detection of *embB306* mutations in ethambutol (EMB) resistant MTB strains. Although a number of studies have been carried out to evaluate the performance of MAS-PCR assay (11, 12), to the best of our knowledge its utility is still awaiting to be explored in Indian set up.

The aims of the present study were twofold: first, to observe the type and frequency of *rpoB* and *katG* mutations in RIF and INH resistant isolates circulating in Northern India and second, to appraise clinical efficacy of MAS-PCR assay in comparison with conventional proportion method (PM) and DNA sequencing for the detection of RIF-resistant and INH-resistant TB strains in a low resource setting.

PATIENTS AND METHODS

Settings

Study was conducted in Department of Microbiology, Institute of Medical Sciences, Banaras Hindu University (BHU). Sir Sundar Lal (SS) Hospital, a tertiary-care hospital of BHU, has a vast catchments area, being the only tertiary care hospital in North Eastern Uttar Pradesh (UP) providing medical cover to more than 15 crore of population of Eastern UP, Western Bihar, adjoining areas of Madhya Pradesh, and Nepal. MTB isolates ($n = 354$), included in the present study were isolated, during January 2008 to January 2010. It included samples from both inpatients and outpatients.

Specimens/Mycobacterial Strains/Phenotypic DST

Pulmonary (85.72%) and extrapulmonary (14.28%) specimens from 3,746 clinically suspected TB patients were collected. Extrapulmonary specimens included pus, endometrial tissue, pleural fluid, lymph node aspirates, urine, and cerebrospinal fluid. Three hundred fifty-four

MTB strains were isolated, identified, and subjected to indirect DST according to the gold standard PM with the recommended critical concentrations of 40 $\mu\text{g/ml}$ for RIF, 0.2 $\mu\text{g/ml}$ for INH, 2 $\mu\text{g/ml}$ for EMB, and 4 $\mu\text{g/ml}$ for streptomycin (STR; (13, 14). H37Rv (ATCC 27294) and a known MDR strain were used as negative and positive controls, respectively.

MAS-PCR Assays for Detection of RIF and INH Resistance Determinants

DNA was isolated as described by van Embden et al. (15) with minor modifications. A two-step MAS-PCR assay was performed to detect mutations at *rpoB* codons (516, 526, and 531) and *katG* codon 315 using 354 purified MTB DNA. Two outer (forward and reverse) and three inner forward primers were used for three MAS-PCRs targeting three different codons of the *rpoB* gene while two outer and one inner reverse primers were used for *katG315* MAS PCR (Table 1). The compositions of reaction mixtures and PCR conditions for each of three different *rpoB* codons and *katG315* codon were as described by Mokrousov et al. (16, 17) with few modifications. Briefly, for *rpoB* MAS-PCR each 25- μl reaction mixture contained 0.5- μl purified DNA sample, MgCl_2 (2.5 mM for *rpoB526*- and *rpoB531*-PCR or 1.5 mM for *rpoB516*-PCR), 1 U of recombinant *Taq* DNA polymerase (Bangalore Genei, Bangalore), 200 μM concentrations of each of the deoxynucleoside triphosphates (dNTPs), outer forward (5 pmol), and outer reverse primer (50 pmol for *rpoB526*- and *rpoB531*-PCR, or 10 pmol for *rpoB516*-PCR) and one of allele-specific inner forward primers R4 (50 pmol) or R5 (50 pmol), or R3 (15 pmol). The reactions of *rpoB526*-PCR and *rpoB531*-PCR were performed under the following conditions: initial denaturation at 96°C for 3 min; five cycles of 95°C for 45 sec, 62°C for 50 sec, and 72°C for 20 sec; five cycles of 95°C for 40 sec, 60°C for 50 sec, and 72°C for 20 sec; 20 cycles 94°C for 50 sec, 58°C for 40 sec, and 70°C for 20 sec; and final elongation at 72°C for 3 min. The conditions of *rpoB516*-PCR were as follows: 96°C for 3 min; 30 cycles of 95°C for 50 sec, 65°C for 45 sec, and 72°C for 20 sec; and 72°C for 3 min. For *katG* 315 MAS-PCR, each 30- μl reaction mixture contained 0.5 μl purified DNA sample, 1.5 mM MgCl_2 , 1 U of recombinant *Taq* DNA polymerase (Bangalore Genei), 200 μM concentrations of each of the dNTPs, outer forward (30 pmol) and outer reverse primer (40 pmol, and one of allele-specific inner reverse primer (30 pmol). The PCR conditions are as follows: initial denaturation at 96°C for 3 min; five cycles of 95°C for 1 min, 68°C for 1 min, and 72°C for 30 sec; five cycles of 95°C for 1 min, 66°C for 40 sec, and 72°C for 30 sec; 20 cycles 94°C for 1 min, 64°C for 40 sec, and 72°C for 30 sec; and final elongation at 72°C for 3 min.

TABLE 1. Description of Primers for the Amplification of *rpoB* and *katG* Gene Segments and Nucleotide Changes Responsible for RIF and INH Resistance

Genes	Primer	Codons/ H37Rv sequence	Nucleotide change (amino acid change)	Sequence (5'-3')	Size (bp)	Position (H37Rv)	Reference (Primers)
<i>rpoB</i>	Outer F (R1)			GTC GCC GCG ATC AAG GA	249	1252–1500	Mokrousov et al., 2003
	Outer R (R2)			TGA CCC GCG CGT ACA C			
	Inner F (R3)	516/GAC	GAC → GTC or TAC (Ser → Leu or Trp)	GCT GAG CCA ATT CAT GGA	214	1287–1500	
	Inner F (R4)	526/CAC	CAC → CGC or GAC or TAC or AAC or GGC (His → Arg or Asp or Tyr or Asn or Gly)	GTC GGG GTT GAC CCA	181	1320–1500	
	Inner F (R5)	531/TCG	TCG → TTG or TGG (Ser → Leu or Trp)	ACA AGC GCC GAC TGT C	167	1334–1500	
<i>katG</i>	Outer F (K1)			GCA GAT GGG GCT GAT CTA CG	435	669–1103	Mokrousov et al., 2002
	Outer R (K2)			AAC GGG TCC GGG ATG GTG			
	Inner R (K3)	315/AGC	AGC → ACC & ACA (Ser → Thr)	ATA CGA CCT CGA TGC CGC	293	669–961	
	Inner R (K4)	315 ^a ACC	AGC → ACC (Ser → Thr)	ATA CGA CCT CGA TGC CGG	293	-	Lavender et al., 2005
	Inner R (K5)	315 ^a ACA	AGC → ACA (Ser → Thr)	ATA CGA CCT CGA TGC CTG	293	-	

^aFor mutant allele; F, forward; R, reverse.

To check the specificity and reproducibility of *katG*315 MAS-PCR assay, a modified MAS-PCR assay (12), which detected mutated alleles instead of wild-type alleles, was performed. Those isolates recognized as not having a wild-type *katG* codon 315 (AGC) were then screened using K4 and K5 mutant allele-specific primers (Table 1) to detect the presence of *katG*315 ACC or ACA mutations, respectively.

After amplification, the amplicon were analyzed by 1.5% agarose gel electrophoresis. Isolate with wild-type codon (either *rpoB* 531, 526, 516, or *katG* 315) produced two PCR products while those having mutation at respective codon produced only one PCR product (Supporting information Fig. 2 file 1).

DNA Sequence Analysis

To verify point mutations (single, double, and triple) detected by MAS-PCR assays and to analyze the samples having discrepancy between the results of PM and MAS-PCR assays, 34 (14 susceptible and 20 resistant), each of 249 bp *rpoB* and 435 bp *katG* preamplified fragments were randomly selected for DNA sequence analysis. The amplified DNA fragment was purified using a HiYield™ Gel/PCR DNA Extraction Kit (Real Biotech Co., Taipei County, Taiwan) and DNA sequencing was performed by using a BigDye Terminator v3.1 cycle sequencing reaction

kit (Applied Biosystems, Foster City, CA) in an ABI prism 3130 Genetic Analyzer Machine. DNA sequence analysis and comparisons of *rpoB* and *katG* were carried out with Chromas V.2.3 and ClustalV programs.

Ethical Issues

The study was approved by the ethical committee of the Institution.

RESULTS

Clinical Characteristics

In our study, of 3,746 specimens, 366 were culture positive among which 354 (96.72%) were tubercle and 12 (3.39%) were nontubercle bacilli. Of 354 tubercle bacilli, 13 (3.67%) were from extrapulmonary and 341 (96.33%) were from pulmonary TB (PTB) cases. Further, new and retreated cases were 230 (64.97%) and 124 (35.03%), respectively. It was observed that 66% cases were males and 34% females among culture positive subjects. Most cases of PTB were in the age group of 21–40 years. Extrapulmonary TB was documented mostly in the age group of <10 years. DST for all four first-line anti-TB drugs was performed, i.e., RIF, INH, STR, and EMB. Among all MTB isolates included in this study, 117, 129, 123, and 113

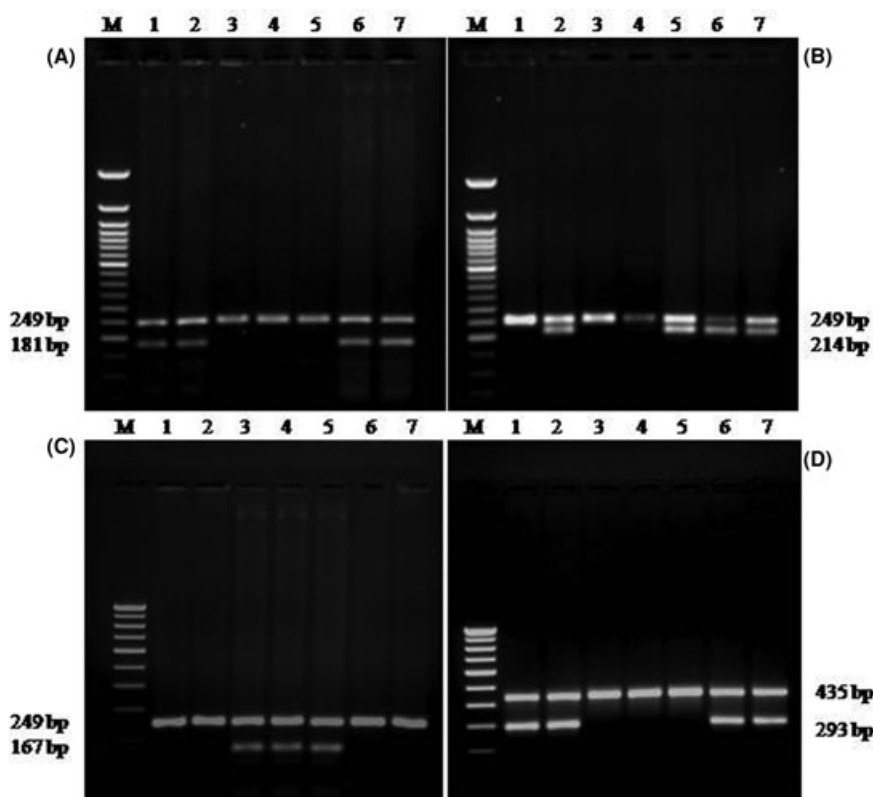


Fig. 1. Agarose gel electrophoresis patterns of MAS-PCR assays: (A) *rpoB526* analysis: Lanes: 3-5, and 1,2,6 strains with *rpoB526* mutant and susceptible alleles respectively; Lane 7: H37Rv. (B) *rpoB516* analysis: Lanes: 1, 3, 4, and 2,5,6 strains with *rpoB516* mutant and susceptible alleles respectively; Lane 7: H37 Rv. (C) *rpoB531* analysis: Lanes: 1, 2, 6,7, and 3,4 strains with *rpoB531* mutant and susceptible alleles respectively; Lane 5: H37Rv. (D) *katG315* analysis: Lanes 3,4,5, and 1,2,6 strains with *katG315* mutant and susceptible alleles respectively; Lane 7: H37Rv. M (Molecular marker): (A & B) 50-bp, (C & D) 100-bp DNA ladder (Bangalore Gene, Bangalore).

were detected resistant to RIF, INH, STR, and EMB, respectively, by PM while 104 strains (29.38%) were detected MDR. Further, among 171 any drug-resistant cases, 72 (42.10%) and 99 (57.89%) were detected primary and acquired drug-resistant cases, respectively.

While performing MAS-PCR assay for detecting mutations in RIF resistance determining region (RRDR) of *rpoB* gene sequence (codons 516, 526, and 531), all 237 RIF-susceptible and 109 of 117 RIF-resistant strains produced two and one PCR products for wild type and mutant allele, respectively (Fig. 1A–C). Single-point mutations resulting in amino-acid change were found in 86 (73.54%) isolates, while 19 (16.24%) and four (3.42%) isolates had double and triple mutations, respectively (Supporting information Table 2). Amino-acid change at codon 531 occurred with the highest frequency (59.83%), followed by those at codon 526 (45.29%) and codon 516 (22.22%).

All 225 INH susceptible strains produced two PCR products, one of which was 293 bp allele specific (Fig. 1D, lanes 1, 2, 6, and 7), implying no mutation in the *katG315* second base and another 435 bp, amplification product of entire region under study. A single MAS-

PCR 435-bp fragment was amplified in 107/129 INH-resistant strains (Fig. 1D, lanes 3–5). Of 129 INH-resistant isolates, 107 (82.94%) had mutations at *katG* codon 315. The wild-type codon, AGC (Ser), was altered to ACC (Thr) in 98 (91.59%) strains and ACA (Thr) in nine (8.4%) strains.

The results obtained by MAS-PCR assays were compared with respect to internationally accepted gold standard PM. Of 117 MTB isolates, which were found to be resistant to RIF by PM, 109 isolates were found to have mutations in RRDR region of *rpoB* gene sequence. None of the strains, which were RIF susceptible ($n = 237$), had shown mutation in the above mentioned gene sequence by MAS-PCR assay. Further, of 129 INH resistant isolates of MTB, 107 isolates were found to have mutations in *katG315* gene sequence but none of the INH susceptible strains ($n = 225$) had shown mutation in the same gene sequence.

The sensitivities and specificities of MAS-PCR assay compared to that of PM were observed to be 93 and 100% for RIF and 83 and 100% for INH, respectively. Further, negative predictive values and positive predictive values were found to be 97 and 100% for RIF and 91 and 100%

for INH, respectively. The accuracy of MAS-PCR assay was appreciable with the standard PM for both the drugs, i.e., INH (94%) and RIF (98%) (Supporting information Table 3 file 3).

Results of DNA sequencing showed that all 14 INH, RIF-susceptible and 16 of 20 INH, RIF-resistant strains produced same results as compared to PM and MAS-PCR assays. Another four INH strains that were detected resistant by PM and susceptible by MAS-PCR showed no mutation in 435 bp *katG* region targeting codon 315. Further, among four selected RIF strains that were detected resistant by PM and susceptible by MAS-PCR; two strains showed no mutation in RRDR while in another two, one showed deletion within RRDR and another mutation at codon 522 (TCG to CCG alteration).

DISCUSSION

Understanding the nature and frequency of mutations associated with drug resistance in MTB in different clinical settings is important for the development and large-scale implementation of rapid, genetics-based assays for the diagnosis of drug resistance.

While performing the PM for DST of MTB isolates in this study, no significant differences between the resistance rates of STR (34.75%), INH (36.44%), RIF (33.1%), EMB (31.92%), and MDR (29.38%) were found. The percentage of drug resistance cases and MDR-TB was observed to be quite high because our samples were obtained from a tertiary care center; sampling bias cannot be ruled out.

The present study revealed that 93.16% of RIF-resistant isolates had *rpoB* gene mutations in RRDR, 73.5, 16.24, and 3.42% of these mutations were single-, double-, and triple-point mutations, respectively. Prevalence of two- to three-point mutations was high in our region with 19.66% (23 of 117) cases. In a study from India (18), the prevalence of double-triple point mutations was found in 48.57% (17 of 35), which was twofold in comparison to our setting. Further, a study from Thailand (11) showed 5.2% and another study from China (19) observed 12.4% prevalence of two- to three-point mutations, which was low in comparison to our findings.

In the present study, among RIF-resistant isolates, 59.83% had mutation in codon 531 of *rpoB* gene, which was similar to other studies; 58% (11), 60% (20), and 59% (21). However, a higher proportion, i.e., 70.5% was reported by Barnard et al. (22). The frequency of mutation at codon 526 was observed among 45.29% of RIF-resistant isolates, which was found to be much higher than that of the studies from four east Asian countries (23), Latvia (24), and India (20), which ranged between 10% and 27%. The rate of mutation at 516 was higher (22.22%) than the range reported elsewhere (20, 22, 25).

However, our observation is in accordance with reports from East Asia (23) and Latvia (24), where higher mutational frequencies in codon 516 (15–32%) along with a predominant Beijing genotype were reported. We have also observed a higher frequency of Beijing genotype in our region (data not shown).

In our study, 107 of 129 INH-resistant isolates (82.95%) had mutations at *katG*315. Similar results were reported in Latvia and Iran (24, 26). However, the frequency of *katG*S315T mutation may vary according to geographical area, even within a single country. A study from India has reported 64% of 70 isolates with the same mutation (27). Similarly, 62% of 79 isolates from Spain and 67% of 37 isolates from the North of Mexico have been reported (28, 29). In partial agreement, in another study, no variability was observed in the mutation frequency of codon 315; a mutation was observed in 100% of 92 isolates from Kazakhstan, Belarus (30). Hence, this mutation needs to be analyzed in a local context, not by international comparison. Furthermore, in our study, the wild-type codon, AGC (Ser), was altered to ACC (Thr) in 97 (77%) strains and ACA (Thr) in nine (7.14%) strains while a study from Australia (12) reported the alteration of wild-type codon, AGC (Ser) to ACC (Thr) in 31 (60%) and ACA (Thr) in three (6%) strains.

Present study observed a very good sensitivities and specificities of MAS-PCR assay compared to that of PM with 93 and 100% for RIF and 83 and 100% for INH, respectively (Supporting information Table 3). Similar results were also observed by Prammananan et al. (11). Further, a study from Turkey (31) showed sensitivity and specificity to be 81.1 and 97.5% for INH, 93.0 and 98.9% for RIF respectively. Moreover, Tho et al. (32) found the sensitivity and specificity of the MAS-PCR to be 83.7 and 100%, respectively for RIF.

We were able to sequence only a limited number of MTB isolates because of low resource set up but that 34 strains are representative strains and succeeded to give the answer of our almost all queries. Similarly, Leung et al. (33) sequenced only 20 MTB isolates (ten INH susceptible and ten INH resistant) to verify the point mutations detected by PCR-RFLP in his setting and found 100% sequence accuracy.

Among those RIF/INH-resistant isolates, in which we were unable to detect the expected mutations employing the above protocol of MAS PCR assay, there might be presence of unusual mutations responsible for the resistance with the same drugs. RIF resistance might be due to mutations in other codons such as 511, 513, 516, 518, 522, and 533 of RRDR of the *rpoB* gene (4). Similarly, INH resistance could be due to mutations in *inhA* regulatory region or *ahpC-oxylR* intergenic region, or *katG* mutations outside of codon 315 (5, 6). Further, simultaneous presence of susceptible and resistant isolates in

culture from a single patient, a condition termed as heteroresistance, could be an obstacle against the sensitivity of molecular drug resistance testing and successive therapy (34). Hence, results indicating susceptibility should be interpreted carefully.

CONCLUSION

It may, therefore, be concluded that in our region the combinations of two- to three-point mutations were prevalent in RIF-resistant MTB strains. MAS-PCR assay is rapid, cost effective, and easy to perform DNA-based protocol for the detection of RIF and INH resistance. Its implementation would be useful for early diagnosis of drug-resistant TB, which may facilitate provision of individualized treatment regimens in patients harboring them.

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