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Rapid detection of drug-resistant *Mycobacterium tuberculosis* directly from clinical specimens using allele-specific polymerase chain reaction assay

Pallavi Sinha[1], Tuhina Banerjee[1], G.N. Srivastava[2] & Shampa Anupurba[1]

Departments of [1]Microbiology & [2]TB & Respiratory Diseases, Institute of Medical Sciences, Banaras Hindu University, Varanasi, India

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***Background & objectives*: Rapid detection of drug resistance in *Mycobacterium tuberculosis* (MTB) is essential for the efficient control of tuberculosis. Hence, in this study a nested-allele-specific (NAS) PCR, nested multiple allele-specific PCR (NMAS-PCR) and multiple allele-specific (MAS) PCR assays were evaluated that enabled detection of the most common mutations responsible for isoniazid (INH) and rifampicin (RIF) resistance in MTB isolates directly from clinical specimens.**

***Methods*: Six pairs of primers, mutated and wild type, were used for the six targets such as codon 516, 526 and 531 of *rpoB,* codon 315 of *katG* and C15-T substitution in the promoter region of *mabA-inhA* using allele-specific (AS) PCR assays (NAS-PCR, NMAS-PCR and MAS-PCR). The performance of AS PCR method was compared with phenotypic drug susceptibility testing (DST).**

***Results*: The usefulness of AS PCR assays was evaluated with 391 clinical specimens (251 Acid fast bacilli smear positive and MTB culture positive; 93 smear negative and MTB culture positive; 47 smear positive and MTB culture negative) and 344 MTB culture positive isolates. With culture-based phenotypic DST as a reference standard, the sensitivity and specificity of the NAS-PCR, NMAS-PCR and MAS-PCR assay for drug resistance-related genetic mutation detection were 98.6 and 97.8 per cent for INH, 97.5 and 97.9 per cent for RIF and 98.9 and 100 per cent for multidrug resistance (MDR).**

***Interpretation & conclusions*: The performance of AS PCR assays showed that those could be less expensive and technically executable methods for rapid detection of MDR-TB directly from clinical specimens.**

Key words Drug susceptibility testing - multiple allele-specific PCR - nested allele-specific PCR - nested multiple allele-specific PCR - rapid diagnosis

The global burden of drug-resistant tuberculosis (TB) and multidrug-resistant TB (MDR-TB), defined as *Mycobacterium tuberculosis* resistant to rifampicin (RIF) and isoniazid (INH), is increasing and has become a major health problem. Globally, among previously treated TB patients, 18 per cent have MDR-TB and 3.5 per cent of all new TB cases are MDR-TB[1]. MDR-TB reduces response to anti-tubercular treatment to

first-line drugs, leads to higher mortality and treatment failure rate and increases periods of dissemination of MTB complex[2]. The RIF resistance increases the percentage of MDR-TB because RIF-resistant (RIF^r) MTB isolates are more likely to be resistant to several other anti-TB drugs[3].

Rapid detection of antimicrobial drug susceptibility pattern in MTB isolates directly from clinical specimens is important for the early administration of proper therapeutic agents to check the development and further dissemination of drug-resistant MTB. In this situation, molecular detection of drug resistance by identifying associated genes that cause mutations will be appropriate for developing a potential rapid molecular drug susceptibility test as an attractive and alternative assay to conventional methods[4-6].

RIF resistance is mostly caused by mutations in *rpoB* gene. Approximately 98 per cent of RIF^r clinical MTB isolates contain point mutations clustered in an 81 bp RIF resistance-determining region (RRDR) between codons 507-533, with the three most common mutations located at codons 516, 526 and 531[7]. In contrast, INH resistance is caused by mutations in various genes, most commonly in *katG* gene and in the promoter region of *inhA*. Different studies have reported that mutations in INH resistant MTB account for 50-95 and 15-34 per cent mutations in *katG315* and *mabA-inhA* promoter region, respectively[7-9]. Different molecular methods have been used to detect specific mutations. However, these methods require specialized instrumentation and are not feasible in resource-poor settings. Thus, there is a need for a rapid, reliable and cost-effective technique for detection of drug resistance in *M. tuberculosis*.

This study was undertaken with the aim to modify the rapid and cost-effective multiple allele specific (MAS) PCR technique to suit the detection of drug-resistant TB directly from clinical specimens, and to evaluate nested MAS (NMAS) PCR assay for direct detection of mutations in *katG315* codon, *rpoB516,* nested allele-specific (NAS) PCR assay for direct detection of mutations in *rpoB526* and *rpoB531* codons and MAS PCR to detect -15C→T mutation in *inhA* promoter region of MTB directly from clinical samples.

Material & Methods

This study was conducted in the department of Microbiology, Institute of Medical Sciences, Banaras Hindu University, Varanasi, India, which is a tertiary care centre of northern region of India. The duration of the study was from May 2013 to March 2015. The study protocol was approved by the institutional ethics committee.

Clinical specimens: A total of 721 clinical specimens were received from clinically suspected pulmonary TB patients that included 656 pulmonary specimens [including 636 sputum speciemns and 20 bronchoalveolar lavage (BAL)] and 65 extra-pulmonary specimens including 20 urine, 15 pus, 15 fine-needle aspirations (FNAs), nine cerebrospinal fluid (CSF), one bone marrow and five pleural fluid. These specimens were obtained from newly diagnosed and previously treated patients. The criteria for patient selection were based on the signs, symptoms, radiological scans, cytology, previous treatment and family history. A total of 721 participants were included, comprising of 429 males and 292 females. Among the 721 participants, 230 were newly diagnosed cases while 302 were previously treated cases and the remaining 189 were unknown. The culture test results were handled in a blinded manner until molecular testing was complete.

The specimens were examined by light microscopy after Ziehl-Neelsen staining. The smear grading was performed using the Revised National Tuberculosis Control Program (RNTCP) recommendations[10]. The clinical specimens received from non-sterile sites [including sputum, pus and BAL] were processed by the conventional modified Petroff's method[11,12]. The samples received from sterile sites (pleural fluid, blood from bone marrow, CSF) were used directly[10]. The sediments were inoculated on Lowenstein-Jensen (L-J) medium in duplicate and incubated at 37°C and inspected weekly for bacterial growth up to eight weeks. The blood from bone marrow and FNA samples were collected aseptically and directly inoculated onto a pair of L-J slants.

Conventional drug susceptibility test: Any suspected growth was confirmed by standard biochemical tests such as nitrate reductase, heat stable catalase and sensitivity to p-nitro benzoic acid (PNB)[13,14]. Drug susceptibility of *M. tuberculosis* isolates to INH (0.2 µg/ml), RIF (40 µg/ml), ethambutol (EMB, 2 µg/ml) and streptomycin (SM, 4 µg/ml) (Sigma-Aldrich, USA) was performed by following standard one per cent proportion method using L-J medium[13,15].

DNA extraction from clinical specimens and MTB isolates: DNA used for the PCR analysis was extracted

from clinical samples and MTB isolates and purified as described by the method of van Embden *et al*[16]. The DNA samples were stored at −20°C.

Allele specific PCR assays for drug resistance detection: These allele-specific PCR assays were performed using specific primers to detect the five most common INH and RIF-associated mutations which target codon 315 of *katG* gene, *inhA* promoter region and codon 516, 526 and 531 of the *rpoB* gene[17-19].

Nested multiple allele-specific (NMAS) PCR for rpoB516 and katG315 codons: NMAS-PCR assay included preliminary amplification of the larger portion of *rpoB, kat*G with outer primers rpoBOF, rpoBOR, katGOF and katGOR (Table I). Each reaction (25 µl) contained 10 pmol (1 µl) of each primers (GeNei, Bengaluru), 0.2 mM (each) dNTPs (2 µl) (GeNei 1U *Taq* DNA polymerase (1 µl) (GeNei 10× PCR buffer (2.5 µl) (GeNei) and 5 µl purified DNA from clinical samples. The first round was performed under the following reaction conditions: initial denaturation 96°C for three minutes; 30 cycles of 95°C for one minute, 65°C for one minute and 72°C for 40 sec and final elongation at 72°C for five minutes.

The NMAS-PCR assay for two codons was performed simultaneously at the same cycling conditions. The reaction mixture for NMAS PCR for *rpoB516* and *katG315* codon contained 10 pmol (1 µl each) of each allele-specific outer forward and inner reverse primers (Table I), 200 µM concentrations of each of dNTPs (2 µl) 1 U *Taq* DNA polymerase (1 µl), 10× PCR buffer (2.5 µl), 5 µl of purified DNA from clinical samples and final volume was maintained by molecular grade water. The reaction conditions were the same as the first-round PCR. For wild-type strains, two fragments of 249 bp and 214 bp were amplified, while a single 249 bp fragment was amplified for mutants in the case of *rpoB* 516 and two fragments of 435 bp and 293 bp were amplified for wild type, while a single 435 bp fragment was amplified for mutants in the case of *katG* 315 codon.

Nested allele-specific PCR (NAS-PCR) assay for rpoB526 and rpoB531 codons: The NMAS-PCR reaction mixture (final volume, 25 µl) for *rpoB*526 and *rpoB*531 comprised of 10 pmol of rpoBOR (1 µl), 10 pmol of one AS inner reverse primer for *rpoB*526 and *rpoB*531 (1 µl), 200 µM concentrations of each of dNTPs (2 µl), 10× reaction buffer (2.5 µl), 1 U *Taq* DNA polymerase (1 µl), 5 µl purified DNA from clinical sample, and final

Table I. Oligonucleotide primers for *rpoB*, *katG* genes and *mabA-inhA* promoter region mutation detection

Oligonucleotide primers for *rpoB* and *katG* genes mutation detection					
Genes	Primers	Codons $H_{37}Rv$ sequence/amino acid	Sequence (5'-3')	Size (bp)	References (primers)
rpoB	rpoBOF		GTCGCCGCGATC AAGGA	249	Mokrousov *et al*, 2003[18]
	rpoBOR		TGACCCGCGCGTACAC		
katG	katGOF		GCAGATGGGGCTGATCTACG	435	Mokrousov *et al*, 2002[17]
	katGOR		AACGGGTCCGGGATGGTG		
rpoB	Outer R		TGACCC GCG CGT ACA C		Mokrousov *et al*, 2003[18]
	Inner F	516 GAC/Asp	GCT GAG CCA ATT CAT GGA	214	
		526 CAC/His	GTC GGG GTT GAC CCA	181	
		531 TCG/Ser	ACA AGC GCC GAC TGT C	167	
katG	Outer F	315 AGC/Ser	GCA GAT GGG GCT GAT CTA CG		Mokrousov *et al*, 2002[17]
	Inner R	315*ACC/Thr	ATA CGA CCT CGA TGC CGG	293	
	Inner R	315*ACA/Thr	ATA CGA CCT CGA TGCCTG	293	
Oligonucleotide primers for mutation detection in *mabA-inhA* promoter region					
Genes	Primers	Codons $H_{37}Rv$ sequence/amino acid	Sequence (5'-3')	Size (bp)	References (primers)
mabA-inhA promoter region	Outer F		ACAAACGTCACGAGCGTAACC	451	Leung *et al*, 2006[19]
	Outer R		GTTGGCGTTGATGACCTTCTC		
	Inner R	-15 C-to-T	TCACCCCGAGAACCTATCG	119	

*For mutant allele ACC/Thr. F, forward; R, reverse

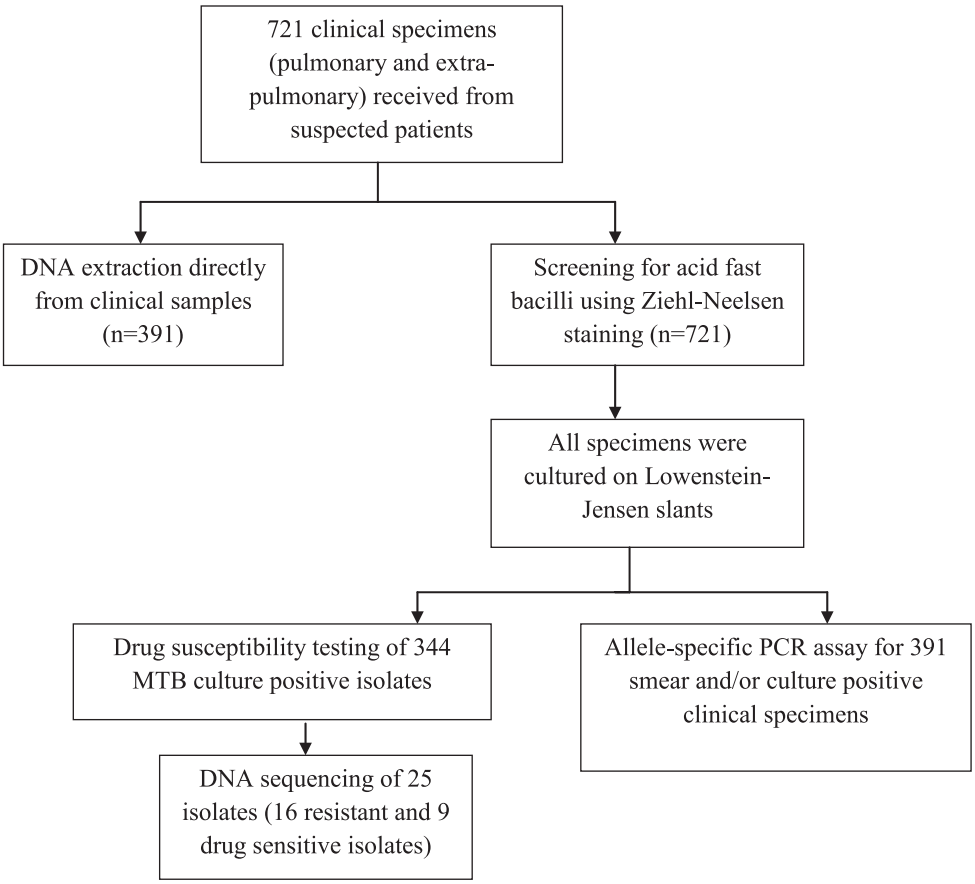

Fig. 1. Flowchart showing available nested multiplex polymerase chain reaction assay, drug susceptibility testing, allele-specific polymerase chain reaction assays and sequencing. MTB, *Mycobacterium tuberculosis.*

volume was maintained by molecular grade water. The reaction was performed in thermocycler (T-100™-Bio-Rad, USA) under the following reaction conditions: initial denaturation at 96°C for three minutes; 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 of 94°C for 50 sec, 58°C for 40 sec and 70°C for 20 sec and final elongation at 72°C for three minutes. The amplified fragments (5 µl) were electrophoresed in 1.5 per cent agarose gels and visualized under UV light. For wild-type strains, three fragments of 249 bp, 181 bp and 167 bp were amplified, while a single 249 bp fragment was amplified for mutants in the case of *rpoB526* and *rpoB531*.

Multiple allele-specific (MAS) PCR for mabA-inhA promoter region: MAS-PCR was performed in 25 µl reaction mixture containing 2 µl 200 µM concentrations of each of dNTPs, 1 U *Taq* DNA polymerase and 10 pmol of each primers designed (Table I) by Leung *et al*[19]. The amplification was performed using the following cycling conditions: initial denaturation at 96°C for three minutes; five cycles of 95°C for one minute, 68°C for one minute and 72°C for 30 sec; five cycles of 95°C for one minute, 66°C for 40 sec and 72°C for 30 sec; 20 cycles of 94°C for one minute, 64°C for 40 sec and 72°C for 30 sec and final elongation at 72°C for three minutes. For wild-type strains, two fragments of 451 bp and 119 bp were amplified, while a single 451 bp fragment was amplified for mutants.

DNA sequencing and analysis: To validate point mutations detected by AS-PCR assays and to analyze the samples having discrepancy between the results of drug susceptibility testing (DST) and AS-PCR assays, 15 (5 susceptible and 10 resistant) each of 249 bp *rpoB* and 435 bp *katG* pre-amplified fragments were randomly selected for DNA sequence analysis. DNA sequencing was performed by Genome Xcelris, Ahmadabad using Sanger sequencing method. The data obtained were compared with sequences from the NCBI database using the alignment tool (Clustal Omega Multiple Sequence Alignment tool, *https://www.ebi.ac.uk/Tools/msa/clustalo/*).

Details of methods for all specimens were depicted in Fig. 1.

Results

Among the 721 clinical samples, 251 (34.8%) were both smear and culture positive, 93 (12.9%) were smear negative culture positive, 47 (6.5%) were smear positive culture negative and 326 (45.2%) were both smear and culture negative. From 721 specimens, 302 (41.9%) were found to be positive for acid-fast bacilli (AFB) smear, whereas 348 (48.3%) were culture positive for *Mycobacteria* spp. and 345 (48.7%) were culture negative while 28 (3.9%) were contaminated. Of the 348 culture-positive isolates, 344 (98.8%) were identified as *M. tuberculosis* while four (1.1%) were identified as non-tubercular mycobacteria.

Drug susceptibility test results by proportion method: Phenotypic DST was performed on 344 *M. tuberculosis* isolates grown on L-J slants. The results showed that 183 samples contained MDR isolates of *M. tuberculosis*. One hundred and ninety eight MTB isolates were resistant to RIF (183 MDR and 15 RIF mono-resistant samples), 208 were resistant to INH (183 MDR and 25 INH mono-resistant samples) and 101 were susceptible to both INH and RIF.

Results of AS PCR assays: A total of 391 microbiologically confirmed (251 Acid fast bacilli smear positive and MTB culture positive; 93 smear negative and MTB culture positive; 47 smear positive and MTB culture negative) clinical samples were tested by AS PCR assays. In addition, a total of 344 MTB culture positive isolates were also subjected to AS PCR assays to detect RIF and INH resistance. The three genes, *katG*, *inhA* and *rpoB*, in the 391 microbiologically confirmed clinical specimens and in the 344 MTB isolates were amplified using AS PCR assays (NMAS PCR, MAS PCR and NAS PCR).

Results of AS PCR of microbiologically confirmed clinical specimens (n=391): Of the 391 clinical specimens, 246 [62.9%; 199 MDR, 29 non-MDR INH resistant (INH^r) and 18 non-MDR RIF^r] harboured INH^r and/or RIF^r bacilli. The remaining 145 (37.1%) clinical specimens harboured bacilli susceptible to INH and RIF drugs. Distinct-banding patterns were obtained for different mutation profiles at the five targeted loci (Figs 2 and 3).

Mutations in rpoB gene: Of the 391 microbiologically confirmed samples, 154 (39.4%) harboured RIF-resistant bacilli had a single nucleotide alteration in codon 531, resulting in the amino acid substitution of Ser to Leu (S531L). The second most affected codons were 516, resulting in the amino acid substitution of Asp to Val (D516V) and His to Asp (H526D) at 526 codon, which were found in 16 (4.1%) and 47 (12.0%) samples, respectively (Table II and Fig. 2). It included nine (2.3%) and 16 (4.1%) isolates harboured double mutations in two separate codons, *i.e.* codons 516 and 526 and codons 526 and 531, respectively. One hundred and seventy four (44.5%) specimens containing bacilli had no mutations in the target codons of *rpoB* gene.

Mutations in katG encoding region and inhA promoter region: According to the AS PCR assays results, 180 (46.0%) of the 391 samples harboured INH^r bacilli, had mutations in *katG* gene at codon 315 (*Ser315Thr*) and 18 (4.6%) had Ser-Asn (*Ser315Asn*) (Table II and Fig. 3). Mutations in the *inhA* promoter region were observed in 30 (7.7%) MTB isolates, which had a mutation at position -15 in the *inhA* promoter region (Table II and Fig. 2). Among those with mutation in *inhA* promoter, five had additional mutation in *katG*315 (*Ser315Thr)*. One hundred and sixty three (41.7%) specimens had no mutations in the target codons studied.

Results of AS PCR of culture isolates (n=344)

Analysis of *rpoB* gene for mutations in rifampicin (RIF)-resistant MTB isolates: Mutations in the RRDR of the *rpoB* gene were identified using NAS PCR assay and NMAS PCR assay in 193 (97.5%) of the 198 RIF^r isolates. A single nucleotide alteration in codon 531, resulting in the amino acid substitution of amino acid substitution of Ser to Leu (*Ser531Leu*), was most prevalent and observed in 142 isolates (71.7%). The second most affected codons were 516 (*Asp516Val*) and 526 (*His526Asp*), which were found in 15 (7.6%) and 36 (18.2%) isolates of *M. tuberculosis,* respectively (Table III). Four (2.0%) and six (3.0%) isolates carried double mutations in two separate codons, *i.e.* codons 516 and 526 and codons 526 and 531, respectively. No mutations were detected in the remaining five (2.5%) RIF^r isolates.

One hundred and twenty three of the 146 phenotypically RIF^s isolates showed the expected wild-type AS patterns while three of the 146 non- RIF^r isolates showed a mutant pattern for the *Ser531Leu* (n=2) and *His526Asp* (n=1).

Analysis of mutations in katG encoding region and inhA promoter region: Among 208 phenotypically

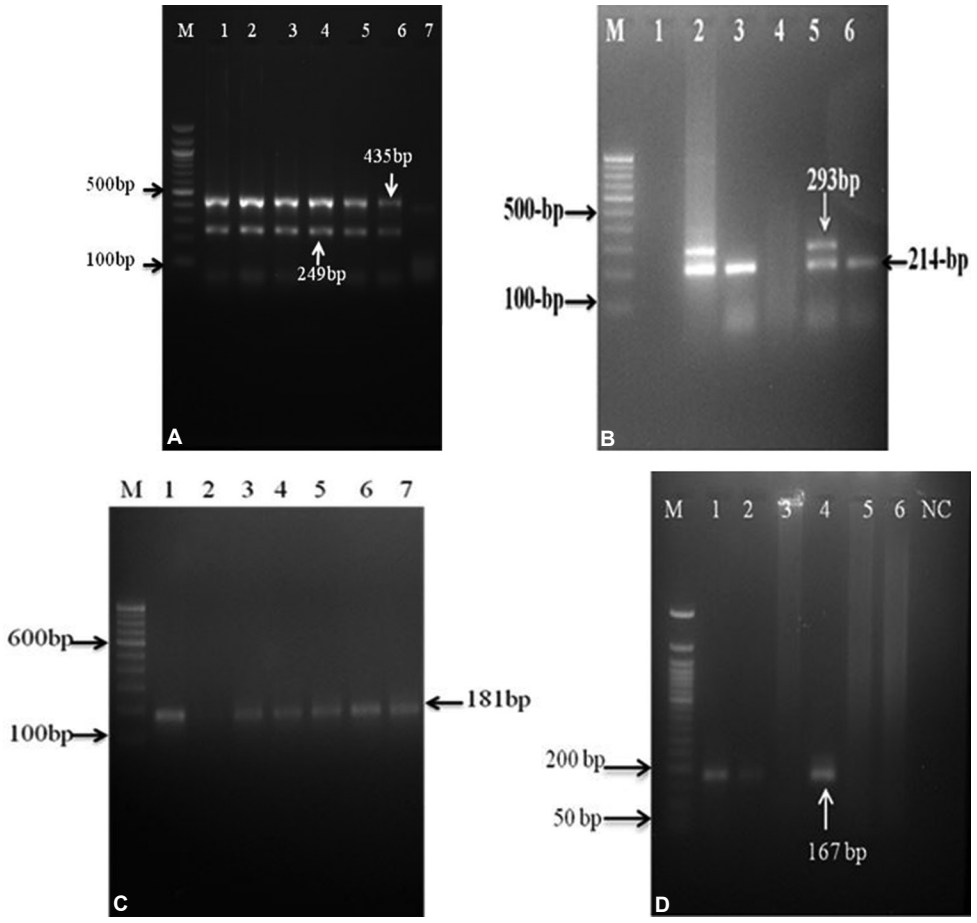

Fig. 2. Gel images of allele-specific PCR assays with clinical specimens DNA preparation: (**A**) First step nested multiple allele-specific PCR with outer *rpoB* and *katG* derived primers. M- 100 bp ladder; lane 1- H_{37}Rv strain; lanes 2 to 6- strains with outer region of codon *rpoB* and *katG* genes; lane 7- negative control. (**B**) Analysis of *rpoB516* codon and *katG315* codon by nested multiple allele-specific PCR assay. Lane 1- negative control ; lane 2- H_{37}Rv strain; lane 3- isolate with *katG315* mutant allele (AGC-ACC); lane 4 - isolate with *rpoB516* mutant allele (GAC-GTC, TAC and GGC) and *katG315* mutant allele (AGC-ACC); lane 5- isolate with *rpoB516* wild-type allele and *katG315* wild-type allele; lane 6- isolate with *rpoB516* wild-type allele and *katG315* mutant allele (AGC-ACA); M- 100 bp DNA ladder; (**C**) Analysis of *rpoB526* codon by nested allele-specific-PCR assay. Lane 1- H_{37}Rv strain; lanes 2 to 7- isolates with *rpoB526* wild-type allele; lane 3- isolate with *rpoB526* mutant allele (CAC-GAC, TAC and CTC); (**D**) Analysis of *rpoB531* codon by nested allele-specific PCR assay. M- 50 bp DNA ladder; lane1- H_{37}Rv strain; lanes 2 and 4- isolates with *rpoB531* wild-type allele; lanes 3, 5 and 6- isolates with *rpoB531* mutant allele (TAC-TCG and); NC, negative control.

INH^r isolates, 177 (85.1%) had *katG* mutations, the vast majority (169; 81.2%) of which was the commonly described substitution *katG* (*Ser315Thr*) (Table III). Eight isolates had Ser to Asn (*Ser315Asn*) substitution at *katG* position 315. Mutations in the *inhA* promoter region were observed in 28 (13.5%) INH^r isolates, which had a mutation at position -15 in the *inhA* promoter region. Among those with mutation in *inhA* promoter, five had additional mutation in *katG315*. Three INH^r (1.4%) isolates had no mutation at *katG* codon 315 and *mabA-inhA* C-15 position.

One hundred and thirteen of the 136 phenotypically INH^s isolates showed the expected wild-type AS patterns while three of the 136 non-INH showed a mutant pattern for the *katGSer315Asn* and -15 position of the *mabA-inhA* promoter region.

DNA sequence analysis: Results of DNA sequencing showed that all nine INH, RIF-susceptible and eight of 16 INH, RIF^r isolates produced same results as compared to phenotypic method and AS-PCR assays. Another three isolates that were detected INH^r by phenotypic method and susceptible by AS-PCR showed no mutation in 435 bp *katG* region targeting codon 315. These INH^r isolates had mutations at *katG* Gly299Ser and *katG* Gln295Ser codons. Another five RIF^r isolates (susceptible by AS PCR) had mutation in *rpoB* 522 (TCG →TGG, Ser→Trp),

511 (TCG→TGG, Ser→Trp) and 533 codon (CTG→CCG, Leu→Pro).

Sensitivity and specificity: The sensitivity and specificity of the NAS-PCR, NMAS-PCR and MAS-PCR for detecting RIF and INH resistance were assessed using conventional DST results as a reference. When compared to DST, the sensitivity and specificity of AS PCR assay for INH resistance were determined to be 98.6 and 97.8 per cent, for RIF resistance were determined to be 97.5 and 97.9 per cent and for MDR were determined to be 98.9 and 100 per cent, respectively (Table IV).

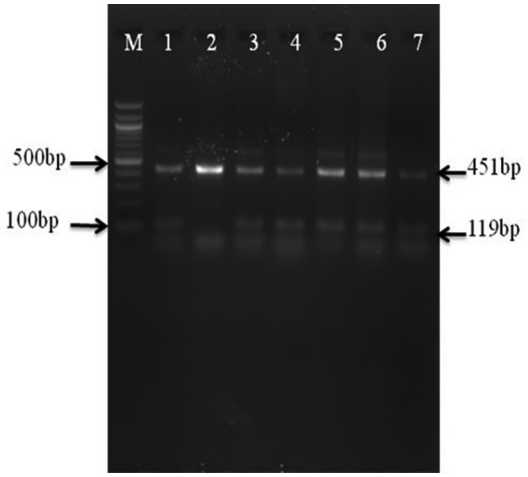

Fig. 3. Gel image of *mabA* single step multiple allele-specific PCR assay with clinical specimens DNA preparation: Analysis of *mabA-inhA* promoter region by multiple allele-specific PCR assay M-100 bp DNA ladder; lane 1- H_{37}Rv strain; lane 2- isolate with a-15 C-T substitution in the promoter region of the *inhA* gene; lanes 3-7- isolates with the *mabA-15* wild-type allele.

Discussion

Anti-TB drug resistance causes a significant risk to human health, which usually develops due to chromosomal mutations in drug targets in *M. tuberculosis* genes[7]. The AS PCR assays offer a rapid, sensitive and cost-effective molecular techniques to accurately screen the most common mutations associated with resistance to RIF and INH. These provide the multiple quality assurance to control for false-negative results due to lack of amplification and especially useful for direct analysis of human samples. Due to PCR inhibitors, the quality and quantity of DNA from clinical specimens may be poor, and the first step PCR is necessary to generate a sufficient template for subsequent AS PCR assay[20].

Resistance mutations observed in a majority of RIF^r isolates (>95%) were due to small deletions or insertions within an 81-base pair RRDR of the *rpoB* gene, between codons 507 and 533[9] and that most of these mutations occurred exclusively at codons 516, 526 and 531. In this study, mutations in *rpoB* region were found in 97.5 per cent of RIF^r isolates using AS PCR assays. The most frequently mutated codon in our study was codon 531 followed by mutations at codons 526 and 516, which was similar to those reported in clinical isolates from India[21-23]. Panama[24], China[25], Pakistan[26], Nepal[4] and other geographical regions[17,18,27]. Café Oliveira *et al*[5] from Brazil showed 62.8 and, 7 per cent mutations at codons

Table II. Distribution of mutations in *rpoB*, *katG* and *inhA* promoter region of 391 resistant *M. tuberculosis* isolates from clinical specimens

Drugs	DNA target	Nucleotide change	Amino acid change	Number of isolates with mutation (n=391) (%)
RIF (n=217)	*rpoB516**	GAC→GTC	Asp→Val	16 (4.1)
	*rpoB526**	CAC→GAC	His→Asp	47 (12.0)
	*rpoB531**	TCG→TTG	Ser→Leu	154 (39.4)
	rpoB516 and *rpoB526*	GAC→GTC and CAC→GAC	Asp→Val and His→Asp	9 (2.3)
	rpoB526 and *rpoB531*	CAC→GAC and TCG→TTG	His→Asp and Ser→Leu	16 (4.1)
	$^{\$}$Wild type			174 (44.5)
Isoniazid (n=228)	*katG 315**	AGC→ACC	Ser→Thr	180 (46.0)
	*katG 315**	AGC→AAC	Ser→Asn	18 (4.6)
	*inhA-15**	C→T	NA	30 (7.7)
	*katG*315 and *inhA-15*	AGC→ACC and C→T	Ser→Thr NA	5 (1.3)
	Wild type$^{\$}$			163 (41.7)

*Including double and triple mutations; $^{\$}$There were no mutations in *katG 315* codon (*Ser315Thr* and *Ser315Leu*) and *inhA* promoter. NA, not applicable; RIF, rifampicin

Table III. Distribution of mutations in *rpoB*, *katG* and *inhA* promoter region in 344 *M. tuberculosis* (MTB) isolates

Drugs	DNA target	Nucleotide change	Amino acid change	RIF^r/INH^r (%)	RIF^s/INH^s (%)
RIF (n=198)	*rpoB516**	GAC→GTC	Asp→Val	15 (5.5)	0
	rpoB526*	CAC→GAC	His→Asp	36 (15.1)	1
	rpoB531*	TCG→TTG	Ser→Leu	142 (71.7)	2
	rpoB516 and *rpoB-526*	GAC→GTC and CAC→GAC	Asp→Val and His→Asp	4 (2.0)	0
	rpoB526 and *rpoB531*	CAC→GAC and TCG→TTG	His→Asp and Ser→Leu	6 (3.0)	0
	Wild type$^\$$			5 (2.5)	143
Isoniazid (n=208)	*katG 315**	AGC→ACC	Ser→Thr	169 (81.2)	2
	katG 315*	AGC→ACG	Ser→Asn	8 (3.8)	0
	inhA-15*	C→T	NA	28 (13.5)	1
	katG315 and *inhA-15*	AGC→ACC and C→T	Ser→Thr and NA	5 (2.4)	0
	Wild type$^\$$			3 (1.4)	133

*Including double and triple mutations, $^\$$There were no mutations in *katG 315* codon (*Ser315Thr* and *Ser315Leu*) and *inhA* promoter. NA, not applicable; RIF, rifampicin; INH, isoniazid

Table IV. Sensitivity and specificity of allele-specific PCR assays and drug susceptibility testing for detecting resistance to isoniazid and rifampicin among 344 *M. tuberculosis* clinical isolates

NAS PCR	DST results		Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
	Number of resistant	Number of susceptible				
INH resistance						
Detected	205	3	98.6	97.8	98.6	97.8
Not detected	3	133				
RIF resistance						
Detected	193	3	97.5	97.9	98.5	96.6
Not detected	5	143				
MDR mutations						
Detected	181	0	98.9	100	100	98.7
Not detected	2	161				

DST, drug susceptibility testing; NAS, nested allele-specific; PPV, positive predictive value; NPV, negative predictive value

531 and 526, respectively. Five phenotypically RIF^r isolates have no mutations in studied *rpoB* codons using AS PCR assays, similar to those reported previously[4,28]. This indicated that most of the RIF^r isolates could be rapidly found by screening of the most common genetic mutations in RRDR region of *rpoB* gene, even though it was necessary to detect the presence of isolates lacking mutations at studied codons.

Earlier studies designated that INH resistance was mediated by mutations in several genes, most commonly *katG*, particularly in codon 315, and the promoter region of *inhA*[4,6,7,29,30]. Accordingly, we found that 85.1 per cent of phenotypically INH^r clinical isolates had point mutations in *katG* gene, and the frequencies were similar to those reported by other researchers[4]. Similarly, 77.6, 71.4, 79, 55.8 and 41.9 per cent of INH^r isolates in India[6], South Africa[31], Pakistan[22], China[32] and Brazil[5], respectively, have been reported for harbouring mutation at the same genetic location. In the present study, 28 isolates had C to T transition at point -15 for *inhA* promoter region reported to be associated with INH resistance as described by Poudel *et al*[4], and mutation frequency in *inhA* was also comparable with other studies showing frequencies varying from 10 to 34 per cent[5,9,26,29,33]. The three phenotypically INH^r isolates had no mutations in *katG* 315 codon and *inhA* promoter region and confirmed that resistance in these isolates could be due to mutations present in other codons of *katG* gene[4,30].

Three phenotypically RIF susceptible isolates have mutations at *rpoB*526 (n=1) and *rpoB*531 (n=2) codons. Similarly, three phenotypically INH

susceptible isolates have mutations at *katG*315 codon (n=2) and at point -15 *inhA* promoter region. It could be possible that these isolates comprised a heterogeneous population of organisms with both wild-type and mutated alleles in the *rpoB*, *katG* and *inhA* promoter region encoding gene, leading to amplification of the corresponding mutated alleles PCR product.

Thirumurugan *et al*[34] have detected the RIF resistance in 127 MTB clinical isolates in Puducherry, South India. In this study, distinct PCR banding patterns were observed for different mutation profiles, and the correlation between MAS-PCR results and phenotypic drug susceptibility test was 96.7 per cent[34]. The major obstructions for utilization of MAS-PCR method in case of clinical samples is the presence of lower amount of mycobacterial DNA in specimens. According to Chia *et al*[24], MAS-PCR method required as high as 20 ng of template DNA per 25 µl of reaction mixture for successful amplification, whereas Vadwai *et al*[35] could not utilize single-tube MAS-PCR assay when amount of DNA was low resulting in amplification failure due to increased competition among primers. To solve this problem, in this study, nested AS-PCR assay was used in which the rationale of first-round PCR was to increase the amount of template DNA for second-round PCR from clinical specimens[20].

The NAS-PCR assay demonstrates multiple quality assurance to check false-negative results due to lack of amplification. This assay is predominantly helpful for direct analysis of human samples, and a wild-type strain (H_{37}Rv) included in each run as a positive control for amplification of the allele-specific fragment. Thus, the absence of a wild-type AS fragment in the tested strain is considered to indicate the presence of mutation and hence a drug-resistant phenotype.

A major limitation to molecular genetic detection of drug resistance by any technique is that such tests generally only detect known mutations. The sensitivity and specificity of a molecular detection assay may vary when being used in different geographic regions, depending on the presence of genetic mutations targeted by a molecular detection assay in the drug-resistant *M. tuberculosis* isolates. Though the molecular methods cannot completely replace culture-based method, but will allow more rapid and decentralized detection of drug resistance and may successfully complement conventional methods.

AS-PCR assay might be a practical and relatively cost-effective molecular method for rapid detection of considerable proportion of RIF^r, INH^r and MDR-TB directly from clinically confirmed samples in India and other developing countries with resource-poor settings.

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For correspondence: Dr Shampa Anupurba, Department of Microbiology, Institute of Medical Sciences, Banaras Hindu University, Varanasi 221 005, Uttar Pradesh, India
e-mail: shampa_anupurba@yahoo.co.in