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Detection of Isoniazid-Resistant Clinical isolates of *Mycobacterium tuberculosis* from India using Ser315Thr marker by Comparison of molecular methods

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ABSTRACT

In this study, Substitution at codon Ser315 of *katG* gene, a reliable marker for isoniazid (INH) resistance was analyzed and compared by three molecular methods such as DNA sequencing, polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) and PCR-single strand conformation polymorphism (PCR-SSCP) in 105 phenotypically resistant isolates obtained from various parts of India. Out of the 105 resistant isolates, 64 (61%) were found to be resistant by DNA sequencing, 54 (51%) by PCR-RFLP and 57 (54%) by PCR-SSCP methods. The results obtained using PCR-SSCP and PCR-RFLP methods were compared with those from DNA sequencing (gold standard). The sensitivity and specificity of PCR-RFLP were 84% and 100% respectively and corresponding values for PCR-SSCP method were 89% and 95% respectively. The study has shown the comparison of the simple, rapid and cost effective methods with DNA sequencing targeting codon Ser315 of *katG* gene and suggests that PCR-RFLP and PCR-SSCP may be performed as alternative inexpensive methods in settings with a high prevalence of INH-resistant *M. tuberculosis* strains where sequencing cannot be afforded.

1. Introduction

Tuberculosis (TB) remains as a major public health problem and leading cause of mortality. The emergence of drug-resistant strains of TB poses threat to the TB control program. Particularly, multi drug-resistant TB (MDR-TB) defined as strains which are resistant to the two most potent anti-TB drugs; isoniazid (INH) and rifampicin (RIF) have caused a deep concern globally. At present, the condition is still complicated by the outbreak of extensive drug resistant (XDR-TB) defined as MDR-TB that is resistant to second-line TB drugs-fluoroquinolones and at least one of three injectable aminoglycosides-Capreomycin, Kanamycin or Amicacin. However, isolates of *M. tuberculosis* resistant to INH are seen with increasing frequency (1 in 10^6) compared to other drugs (Nachega and Chaisson, 2003).

INH has been extensively used as the frontline anti-TB drug and a choice for chemoprophylaxis. It is proposed that INH enters the cell as a prodrug by passive diffusion and is activated by catalase-peroxidase, encoded by *katG*, (Zhang et al., 1990) to generate free radicals, which transforms INH into its active form. Studies have shown that *inhA,* encoding enoyl-acyl carrier protein (ACP) reductase and *kasA,* encoding keto acyl-ACP synthase, are important intracellular targets for activated INH involved in elongation of fatty acids intermediate in the biosynthetic pathway of mycolic acids (Banerjee et al., 1994; Mdluli et al., 1998). In addition,

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mutations in the *oxyR-ahpC* intergenic region have been identified in INH-resistant (INH^r) isolates (Sreevatsan et al., 1997). Of all the other genes, 50-90% of INH^r isolates encounter mutations in *katG*.

The most prevalent mutation found to occur in *katG* gene was Ser315Thr (S315T), serving as a reliable biomarker for the detection of INH^r (Ramaswamy and Musser, 1998). The appearance of this mutation was most frequent among the MDR strains (Marttila et al., 1998). However, this mutation was also reported to be associated with intermediate or high levels of resistance to INH (1 to 10 µg/ml) (van Soolingen et al., 2000). Compared to other resistance-conferring mutations in *kat*G, S315T was found to result in near-normal catalase-peroxidase activities and maintains the levels of virulence at the same time conferring resistance to INH (Pym et al., 2002).

Worldwide INH resistance is more frequently associated with mutations in the *katG* gene. However, little data from India have been reported so far. The present study represents the first to analyze *katG* gene in a total of 105 INH^r isolates from India. Several molecular methods have been described in the last few years, to detect INH resistance in *M. tuberculosis* (Ahmad et al., 2002; Bakonyte et al., 2003; Bang et al., 2006; Herrera-Leon et al., 2005; Hillemann et al., 2007; Kiepiela et al., 2000; Leung et al., 2003; Marin et al., 2004; Mokrousov et al., 2002; Silva et al., 2003; Telenti et al., 1997; Uhl et al., 1996). Unlike other reports, comparison was made in the present study among the genotypic methods to establish the effectiveness and efficiency of rapid, simple and low cost assays, which will have significance in resource-limited settings with a high prevalence of INH^r strains.

The aim of the current study was to detect mutations at codon Ser315 of *katG* gene in INH^r clinical isolates of *M. tuberculosis* from India by comparison of genotypic methods namely PCR-RFLP (Parry et al., 1990) and PCR-SSCP (Orita et al., 1989) with DNA sequencing (Sanger et al., 1977).

2. Materials and methods

2.1. Clinical isolates

A total of 105 INH^r clinical isolates of *M. tuberculosis* were randomly selected from patients (20-60 years) including both the sexes from 2006-2009. Out of 105, 84 isolates were obtained from South India (Tamil Nadu, Andhra Pradesh, Karnataka and Kerala) and 21 isolates were from North India (Assam, Delhi, Goa, Gujarat and Sikkim). Drug susceptibility testing (DST) was performed at National Institute for Research in Tuberculosis (NIRT), Chennai, India. A laboratory reference strain of *M. tuberculosis*, $H_{37}Rv$, was used as the control. All the cultures were coded and subjected to DNA sequencing, PCR-RFLP and PCR-SSCP to identify mutations in *katG* gene.

2.2. Indirect susceptibility test

DST was performed using Lowenstein–Jensen (LJ) medium and INH susceptibility was determined by minimum inhibitory concentration (MIC) method. Briefly, the method involves preparing a 4 mg moist weight per ml culture suspension of the *M. tuberculosis* bacillary mass in a sterile bijou bottle containing 4–5 glass beads (2–3 mm). The bottle was shaken mechanically for 1 min to produce a uniform suspension. Using a 3 mm external diameter nichrome wire loop, a loopful of this suspension was inoculated onto drug-free and drug containing LJ media (1, 5, and >5 mg of INH per liter). All the slopes were incubated at 37°C and the MIC was recorded at the end of 4 weeks. MIC was defined as the lowest concentration of the drug that inhibited the growth of *M. tuberculosis*. 'Growth' on an LJ slope was defined as the presence of 20 or more colonies (WHO 1960). All cultures resistant to INH had an MIC 1, ≥ 5 mg/L, while sensitive cultures had an MIC of <0.2 mg/L. About 59 MDR and 46 non MDR INH^r isolates were included in this study.

2.3. Amplification

Amplification was performed using the mixture containing 1 µl of forward and reverse primers (10 pmol) each, 6 µl of deoxyribonucleoside triphosphates (dNTP) mix (2.5mM), 2.5 µl of 10X PCR buffer, 10-50 ng of template genomic DNA and 1 U of *Taq* DNA Polymerase (Amersham Biosciences, UK). The amplification was performed in a thermal controller (MJ Research, USA) with 30 cycles (1 min at 95°C, 30 sec at 63°C and 1 min at 72°C, followed by a final extension step at 72°C

for 10 min) in the isolated genomic DNA as described elsewhere (Siddiqi et al., 1998). The primers of *katG* gene forward sequence (5'-AAACAGCGGCGCTGGATCGT-3') and reverse sequence (5'-GTTGTCCCATTTCGTC GGGG-3') were used to generate a 209-bp fragment containing the S315T codon (Kiepiela et al., 2000). The amplicons were purified using GFX COLUMN (Amersham Biosciences, UK) according to the manufacturer's instructions.

2.4. DNA Sequencing

Sequencing of the amplicon was carried out using an automated DNA sequencer (ABI Prism 310 Genetic Analyzer-Applied Biosystems, USA), using the above mentioned primers and the Bigdye terminator sequencing kit (Applied Biosystems). To 4 µl of the Terminator ready reaction mix, l µl of the amplified fragment (2–3 ng) and 1 µl of the primer (10 pmol/ µl) were added and the volume was made up to 20 µl using deionised water. The reaction mixture was subjected to cycle sequencing. The samples were vortexed and spun, then heated at 95°C for 2 min and immediately chilled on ice. They were vortexed and spun again and placed on ice, to load onto the DNA sequencer. The data obtained was compared with sequences from the database EMBOSS using the alignment tool via http://www.ebi.ac.uk/emboss/align/. The GenBank accession number for *katG* is X68081.

2.5. PCR-RFLP

Restriction digestion was performed on the amplicon using *MspI* enzyme (New England Biolabs, UK) as previously described in a report (Kiepiela et al., 2000) with slight modifications. The mixture containing 1µl of enzyme, 2 µl of buffer, 10 µl of amplicon was made up to 20 µl using deionised water. The contents were incubated for 1 hr at 37°C and loaded on 10% (39:1) native polyacrylamide gel electrophoresis (PAGE) with 1X Tris-Acetate-EDTA as running buffer.

The electrophoresis was standardized to run for 1 hr at 100 V at 4°C for proper separation. After the run was over, the two plates were separated carefully, gel transferred on to a tray containing ethidium bromide for 15 min and visualized under UV light.

2.6. PCR-SSCP

The amplified PCR fragment was denatured at 98°C for 12 min in the presence of an equal amount of stop buffer (2 mM EDTA, 95% formamide and 0.05% bromophenol blue) with both primers (300 pmols). The reaction mix was snap-cooled on ice and loaded immediately on 10% PAGE. The gel was standardized in varying ratios of acrylamide: bis acrylamide (19:1, 29:1 and 39:1) and optimized at 49:1 ratio. The run was performed in a vertical slab gel apparatus (Bangalore Genei, India) with 1X Tris-Borate-EDTA as the running buffer. The electrophoresis was standardized to run for 2 hrs at 50 V at 4°C. DNA bands in the gel were visualized by silver staining, as described by Ainsworth et al 1999. Briefly, the gel was first treated with 10% ethanol for 5 min followed by 1% nitric acid for 3 min. The gel was then stained with 0.2% silver nitrate solution and 10% formalin for 20 min. The gel was washed with distilled water for 3 min each before exposure to the developing stain (3% sodium carbonate solution containing 10% formalin). The reaction was arrested by adding 10% glacial acetic acid.

2.7. Interpretation of the results

DNA sequencing is considered the gold standard among molecular methods for the detection of mutations associated with drug resistance. Modern sequencing is based on Sanger's method of dideoxy sequencing, which uses dideoxy nucleotides to allow chain termination at each base. In this study, an isolate with mutation in *katG* gene was considered resistant, while an isolate with no mutation in the gene as sensitive. PCR-RFLP, a molecular method, makes use of bacterial DNA-modifying enzymes called as restriction endonucleases (REs). RE recognizes specific sequences and cleaves them at the site whereas it would not recognize if the cleavage site is modified due to point mutation. In this study, an isolate was considered resistant when bands of 79, 52, 46-bp were seen whereas the presence of bands of 79, 67 (46+21) and 52-bp length similar to the wild-type control strain $H_{37}Rv$ were considered as sensitive isolate. PCR-SSCP relies on the fact that single-stranded DNA in solution under certain conditions has a defined secondary structure; the

electrophoretic mobility of the single-stranded DNA is dependent not only on its length and molecular weight, but also on its overall conformation. In this study, a sample showing very close bands was considered resistant while sample displaying distant bands similar to the wild-type $H_{37}Rv$ as sensitive.

2.8. Statistical analysis

DNA sequencing was considered as the gold standard for the analysis. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and kappa values were obtained using SPSS software version 14.0 (SPSS Inc., Chicago). The mean difference, P value, confidence interval (CI) value of difference among the three methods were obtained using Graph Pad Prism software version 4.0 (GraphPad Inc., USA).

3. Results

3.1. DNA sequencing

In this study, the frequency of mutation was higher in MDR (66%) than (58%) in non-MDR INH^r isolates. In addition, a strong correlation was found between the MICs of INH and level of mutation in the isolates. In 38 isolates with high MIC values of ≥ 5 mg/L, the mutation rate was found to be 84% in contrast to 71.6% in the remaining 67 isolates with MIC value of 1 mg/L. An observation from various states of India indicates that, the frequency of mutation in South India was 66% and North India was 47%. No correlation could be made, since the number of North Indian isolates used in this study was fewer.

Among the 105 INH^r isolates, 64 showed mutations, of which, 54 isolates had ACC, 6 had AAC, 3 had ATC and one had CGC substitution. No mutation was detected in the 41 phenotypically resistant isolates by sequencing method.

3.2. PCR-RFLP

Fifty-four resistant isolates (with ACC substitution) were identified as resistant and 10 resistant isolates (with AAC in 6, ATC in 3 and CGC in one isolate identified by DNA sequencing) were identified as sensitive by PCR-RFLP method, out of 64 resistant isolates. The banding patterns for the remaining 41 phenotypically resistant isolates were found to be sensitive similar to DNA sequencing by the presence of AGC. Hence, the sensitivity and specificity of the assay was 84% and 100% respectively. Figure 1 shows a representative PCR-RFLP. The second base change from G to C which created an additional *Msp1* restriction site (C▼CG▲G) in the codon ACC resulted in 79, 52, 46-bp bands compared to the bands of 79, 67 (46+21) and 52-bp length as seen in the wild-type AGC codon with a loss of *Msp1* site. The bands of 79, 67, 52, 46-bp were seen whereas the last three bands of 21, 6, 5-bp were not seen in gel and was determined on the basis of number of restriction sites.

3.3. PCR-SSCP

Of the 64 resistant isolates identified by DNA sequencing, 57 were identified as resistant and 7 were identified as sensitive by SSCP method. Figure 2 shows a representative PCR-SSCP. Out of 57, 54 showed close bands with ACC codon and 3 with ATC codon. Out of the seven, six isolates had AAC and one had CGC at codon 315. The results obtained by PCR-SSCP were compared with the DNA sequencing. The sensitivity, specificity, PPV, NPV and the kappa values of the assay are given in Table 1.

In the present study, the difference in number of isolates for detection of mutation with DNA sequencing vs PCR-RFLP and PCR-SSCP were 10 and 9 (7 for resistant and 2 for sensitive isolates) respectively. The inter-difference between PCR-RFLP and PCR-SSCP was 5 (3 for resistant and 2 for sensitive isolates) (Table 2). There were no observed significant differences ($P<0.05$) between DNA sequencing and PCR-RFLP, SSCP. The (kappa value) agreement for both the methods with sequencing was 82 and 80% respectively (Table 1). 95% CI value of difference for PCR-RFLP vs DNA sequencing was -0.2581 to 0.06762, and for PCR-SSCP vs DNA sequencing the value ranges from -0.2295 to 0.09619. Refer supporting documents for figure. The mean difference between PCR-RFLP and PCR-SSCP was -0.02857 (Tukey's Multiple Comparison Test).

Table 1. Concordance of PCR-RFLP and PCR-SSCP assays with results obtained by DNA sequencing for testing susceptibility of *M. tuberculosis*

	DNA sequencing						
Methods	R	S	Sensitivity	Specificity	PPV	NPV	Kappa agreement
and results	(n)	(n)	(%)	(%)	(%)	(%)	(%)
PCR-RFLP							
R	54	0	84	100	100	80.4	80
S	10	41					
PCR-SSCP							
R	57	2	89	95	96.6	95	82
S	7	39					

Resistant = R; Susceptible = S; PPV = Positive predictive value; NPV = Negative predictive value; n = number of isolates

Table 2. Discrepancy of DNA sequencing with PCR-SSCP and PCR-RFLP assays

Isolate no.	DST	MIC	DNA	sequencing[a]	PCR-SSCP	PCR-RFLP
1	H	>5	AAC	- R	S	S
2	MDR	5	AAC	- R	S	S
3	H	5	AAC	-R	S	S
4	H	1	AAC	-R	S	S
5	MDR	1	AAC	-R	S	S
6	MDR	5	AAC	-R	S	S
7	H	1	ATC	-R	R	S
8	MDR	1	ATC	-R	R	S
9	MDR	5	ATC	-R	R	S
10	H	> 5	CGC	-R	S	S
11	MDR	1	AGC	-S	R	S
12	MDR	1	AGC	-S	R	S

Resistant = R; Susceptible = S; Isoniazid = H; a = variants at codon 315 of *katG*

Table 3. Details of costs of the tests in routine

General Requirement	Minimum Quantity	*Approximate cost in INR	Specific Requirement	Minimum Quantity	*Approximate cost in INR
PCR machine	1	3000	Enzyme (*MspI*)	400U	1370
PCR kit	100[a]	3790	Sequencer machine	1	9,91,153
Primers	2[b]	2420	Sequencing kit	1U	24,794
Transilluminator with CCD camera	1	2,08,176			
Gel tank with power pack [c]	1	6803			
Vertical model tank	1	5205			
Four pipettes	1	15,068			
Waterbath	1	37,260			

Labor costs were not included in this calculation. Costs were estimated in the Indian rupees (INR). *Equipment cost estimates was based upon locally available catalogue prices without including discount and VAT (value added tax) rates. a = Reactions; b = base pairs; U = unit. c = horizontal model

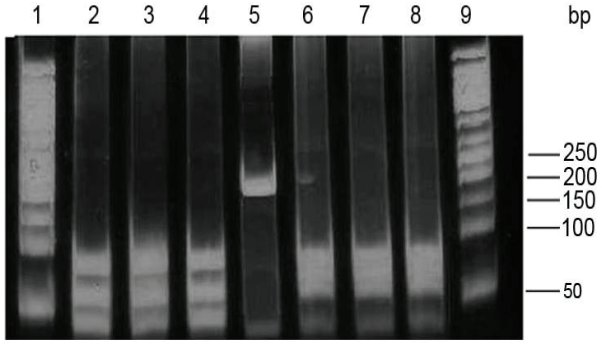

Figure 1. PCR-RFLP of 209 bp amplicon. Lanes: 1,9 are 50 bp DNA ladder, 5, control double stranded DNA; 2-4, resistanat strains, 7 and 8, sensitive strains, 6, $H_{37}Rv$ control strain.

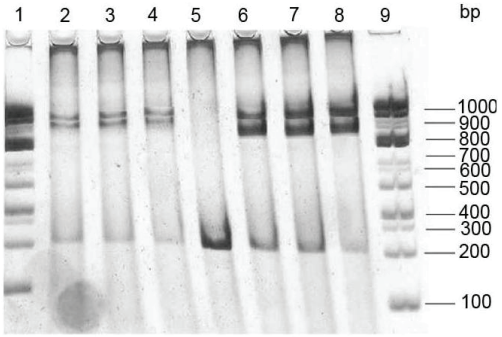

Figure 2. PCR-SSCP of 209 bp amplicon. Lanes: 1,9 are 100 bp DNA ladder, 5, control double stranded DNA; 2-4, resistanat strains, 7 and 8, sensitive strains, 6, $H_{37}Rv$ control strain.

4. Discussion

Out of 105 phenotypically resistant isolates selected on the basis of MIC values, 64 (61%) were found to be resistant and 41 were sensitive by DNA sequencing (genotypic) method; the mutation rate found in this study was consistent with the global pattern (Ramaswamy and Musser, 1998). The discrepancies between the phenotypic (MIC) and genotypic method are mainly due to the fact that the MIC method involves whole cell screening whereas sequencing was done on a segment of the *katG* gene. Although resistant isolates were selected on the basis of MIC values in this study, comparison was not made with phenotypic method because the INH^r phenotype may be owing to mutations in other regions of *katG* (not included in this study) and also genes other than *katG* such as *inh*A, *ahp*C, *kas*A, *ndh*.

The study was focused on *katG* and not other genes such as *inhA, ahpC, kasA* which contributes to >10% of INH resistance, in spite of the fact that sequence analysis was performed in *inhA aphC* and *kasA* for 80, 40 and 25 clinical isolates respectively in our previous study (Nusrath Unissa et al., 2008). Since the current study involves consistent comparison of sequencing with RFLP and SSCP methods on all the isolates revolving around single segment of *katG* gene which was not possible with other genes, as RFLP cannot be performed due to lack of an enzyme which could detect the presence of clinically significant mutation in them. Since 50-90% of INH resistance is attributable to mutation in the *katG* region, other genes were not considered for the study.

In recent years, interest is shown towards those strategies which can be directly employed on clinical or respiratory samples (Bang et al., 2006; Hillemann et al., 2007; Leung et al., 2003; Marin et al., 2004) whereas the present study was based on primary cultures using conventional PCR strategies with principally considering the cost factor. The costs of the test in routine use were calculated based on its suitability for resource poor settings and in regions having high prevalence of INH^r strains where access for sophisticated techniques is unavailable. With the minimum equipment requirements, all reagents and accessories purchased through local distributors. With a mutant and wild-type control, the cost of each test will be in the range of Rs. 50-150 per isolate whereas for sequencing it may cost around Rs. 1500 per isolate. In addition, sufficient space is required for installation of the instrument for DNA sequencing along with the kit cost, whereas in the context of SSCP and RFLP no such space or expenses are required (Table 3).

In *katG* specific PCR-RFLP method, 84% (54/64) of isolates were identified as resistant (ACC) and the remaining 15% (10/64) of resistant isolates were identified as sensitive (AGC). Upon enzymatic digestion, the remaining resistant isolates with mutations other than ACC such as AAC, ATC and CGC substitutions did generate the same length of fragments (3) as sensitive isolates. Compared to others (Ahmad *et al*., 2002; Kiepiela *et al*., 2000; Leung *et al*., 2003; Uhl *et al*., 1996) the present study shows the lower sensitivity of this assay. However, reports (Bakonyte et al., 2003; Herrera-Leon et al., 2005) showed an increase in sensitivity of the assay, using *SatI* enzyme in conjunction with *Msp1* to detect any mutation (AAC and ATC) at codon 315.

PCR-SSCP was used to detect point mutations in the *katG* gene whereby the sensitivities and specificities ranged from 60% to 100% (Kiepiela et al., 2000; Silva et al., 2006; Telenti et al., 1997). Similar results were obtained in the present study, with a sensitivity of 89% and a specificity of 95%. The PPV of the assay was 96.6%. Of the 64 resistant isolates, 57 were identified as resistant and 7 were identified as sensitive by SSCP method. However, the reasons for the misclassification of the resistant isolates as sensitive and two sensitive isolates as resistant are still not clear, and it is assumed that conformation of purine nucleotides viz., A and G is different from pyrimidine nucleotides C and T at the second base of the wild type codon AGC.

Although all the three assays were also performed on 25 phenotypically susceptible isolates, all gave results (no mutations) similar to that of wild-type $H_{37}Rv$, confirming susceptibility genotypically. Statistical comparison cannot be done for this isolates as the assays show no variation, as shown by the 105 phenotypically resistant isolates.

5. Conclusions

All the above assays have an inherent limitation that they were all performed using the primary isolates. The results of the assays were available within 24 hours after the isolation of the primary culture whereas the phenotypic DST testing requires 42 days to return a result. Although, in the present study, sequencing was considered as gold standard with its advantages as rapidity and precise determination of the location and nature of the mutations, the requirement of high infrastructure and economic factor restricts its applicability in resource-limited settings. PCR-RFLP is a robust, economic, and specific method, but requires site for mutant allele detected by the enzyme especially in instances when more than an allele is associated with the mutation. PCR-SSCP analysis have advantages such as apparent simplicity, visual interpretation, non-radioactive means of detection, rapidity and cost-effectiveness, but very often associated with lower sensitivity and specificity. Thus, in the present study, PCR-RFLP showed highest specificity and PCR-SSCP showed greater sensitivity compared to DNA sequencing. Therefore, a combination of both the methods would provide a high degree of specificity and sensitivity making them equivalent to DNA sequencing. This may be of use in resource-limited settings such as India, where required infrastructure is unavailable for sequencing and high prevalence of INH^r *M. tuberculosis* strains occurs. However, newer technologies, especially those that can be performed directly on the specimens themselves as reported already need to be explored further to rapidly identify INH resistance in *M. tuberculosis* in resource-limited settings. Importantly, optimization of molecular methods should significantly shorten the turnaround time for the detection of mutations in clinical specimens without the need for a viable biomass.

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