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Role of *Kat*G Gene in Resistant Buildup Against Isoniazid (INH)–A First Line Anti-TB Drug

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ABSTRACT

The main objectives of the study were to perceive the pattern of drug resistance amongst the isolates of *Mycobacterium tuberculosis*, especially to Isoniazid (INH) resistant strains and to assess either the mutation in *Kat*G is predictive of INH resistance or not. In this way, diagnosis of MDR-TB cases in short time using a molecular based technique may be possible. For this prospect, 24 INH resistant strains on Lewenstein-Jensen (L.J) culture drug sensitivity medium were selected for Single Strand Confirmation Polymorphism (SSCP) analysis to investigate the mutation in *Kat*G gene on molecular level. In this study, out of 24 INH resistant samples only one (4.17%) displayed mutation in *Kat*G gene. Bands of these mutant strains had a detectable change in migration for DNA fragment. Bands from 23 (95.83%) samples did not show any change in migration. So, for the investigation of INH resistance, the use of only *Kat*G gene mutation is not reliable. Other genes like *inh*A, *kas*A, *ahp*C and the genes under study should also be used for the detection of INH resistance.

Key Words: Tuberculosis; MDR-TB; Isoniazid; Resistance; *KatG* gene; Mutation

INTRODUCTION

Currently TB is treated with an initial intensive two months regime multiple antibiotics, Rifampicin (RIF), Isoniazid (INH), Pyrazinamide (PZA), and Ethambutol (EMB) or Streptomycin (SM) to minimize the chances of resistance development against single drug. The next four months, only RIF and INH are administered to eliminate any persisting tubercule bacilli (Anonymous, 1997). INH and RIF, the two most potent anti-TB drugs, kill more than 99% of tubercule bacilli within two months of initiation of therapy (Mitchison, 1985; Iseman & Madsen, 1989).

Isoniazid (INH) was first reported to be an effective anti-tuberculosis drug in 1952, displaying particular potency against *Mycobacterium tuberculosis* (MTB) and *M. bovis* (Bernslein *et al.*, 1952). Mutants resistant to INH have emerged later on (Middlebrook, 1952) and today such mutants account is 26% of clinical MTB isolates in certain cities of the United States (Frieden, 1993). Prevalence of INH resistance has been reported 10% in NWFP (Safi *et al.*, 1994), 7% in Nairobi Kenya (Kibuga, 1996), 15% in India (Trevida & Desai, 1988) and 10-25% in New York (Stoeckie, 1993).

INH is a key drug of TB therapeutic regimens, therefore patients in whom resistance developed to this drug have a poor outlook. In INH resistant strains the mutation in *Kat*G, *inh*A, *Kas*A and *ahp*C genes had been detected, but a small portion of INH resistant strains do not have mutation in these genes, indicating that new undefined genes are also involved in INH resistance. It will be interesting to see if these genes are also identified. This will not only help to understand how INH works but will also facilitate the design of molecular assay for more efficient detection of INH resistant organisms (Zhang & Amalio, 2000).

Some INH resistant strains are associated with the loss of catalase-peroxidase activity (Middlebrook, 1954), and deletion of catalase-peroxidase gene (*Kat*G) correlated with INH resistance in certain MTB isolates (Frieden, 1993). Furthermore transfer of the wild-type *M. tuberculosis* strain's *Kat*G gene on a multicopy plasmid to INH resistant *M. Smegmatis* and *M. tuberculosis* strain confers INH sensitivity (Frieden, 1993). Thus previous studies suggesting that catalase-peroxidase activity is required for INH sensitivity. INH resistance can be due to other factors also (Gopinathan & Saroja, 1981; Stoeckie, 1993).

Several methods have been developed for the determining of a single base change existing in amplified DNA, but they have not been widely used because of time consuming protocols, poor reproducibility, complicated instrumentation and undetected base changes (Ganguly & Prockop, 1990). Because resistance to INH involves changes in the genes those encode catalase peroxidase activity and fatty acid (Mycolic acid) synthesis (*inh*A and *Kat*G genes) thus a simple and rapid screening method can be developed for the detection of these mutations which may be helpful to detect resistance in INH on molecular level. For this purpose DNA sequence analysis, dideoxy fingerprinting (ddF) and Single Strand Conformation Polymorphism (SSCP) can be used. The first and second techniques are either expensive or time consuming, where as the SSCP is very rapid and economical.

So, this study was initiated to know the pattern of drug resistance amongst the isolates of MTB, especially to INH resistant strains. Detection of mutation in the catalase-peroxidase gene (*Kat*G) of MTB was used as an accurate

predicator of INH resistance. The aim of this study was to develop a test, which may be helpful for the detection of INH resistant cases on molecular level.

MATERIALS AND METHODS

A loop of MTB isolate resistant to INH was collected from the growth surface of Lewenstein-Jensen (L.J) culture medium containing INH drug from PMRC, TB research center, Mayo hospital Lahore. DNA was extracted from the strains by mechanical method (Idrees *et al.*, 1998). SSCP-PCR method was used for the detection of mutation in INH resistant strains. The reaction mixture for PCR consisted of 2 µl (20-50 ng) of isolated DNA to amplify 228 bp fragment from *kat*G gene in 18 µl of reaction buffer containing 19 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.2 mM deoxynucleotide Triphosphate (dNTPs), 10 pM of each primer and one unite of Taq DNA polymerase enzyme. After mixing the samples were spun and shifted to thermocycler. The PCR was programmed as follows: after 2 minutes of initial denaturation at 95^0C, 35 cycles were run with 1 minute denaturation at 94^0C, 1 minute of primers annealing at 56^0C and 1 minute of primer extension at 72^0C. After 35 cycles, the final extension was done for 10 minutes at 72^0C. Both positive and negative controls were included in each experiment. The negative control reaction consisted of all the above PCR reagents except the template. SSCP relies on the fact that single stranded DNA or RNA of different sequences exhibit different mobility during electrophoresis in non-denaturing polyacrylamide gels. The strategy of the method was to amplify the 228 bp fragment of *Kat*G gene of resistant strain to INH an anti-TB drug by PCR with specific primers, which were self designed and then to compare the mobility of the denatured DNA with that of the reference fragment of known sequence. Since single point mutation within the sequence leads to a changed running behavior in the gel, this method is very well suited for the detection of mutations in the DNA segment. Single-stranded DNA (ssDNA) exhibits a specific folded structure under non-denaturing conditions, which is determined by intra molecular interactions and thus by the sequence. The changes in the DNA sequence by mutation results in the change of folded structure. Thus, in the SSCP analysis, the mutation was determined by the mobility shift of the single stranded DNA (ssDNA).

To optimize the conditions of non-denaturing polyacrylamide gel electrophoresis (PAGE), different percentages of TBE buffer and DNA, starting and running voltages, buffer temperatures and running time was tested. 1 µl of PCR product was mixed with 6 µl SSCP dye, and kept at 37°C for 1 hour. The samples were then heat denatured at 95°C for 5 minutes in a thermal cycler and immediately chilled on ice. The samples were electrophoresised in a 20% (39:1: acrylamide: bis-acrylamide) non-denaturing PAGE (8.0 cm X 8.0 cm X 1 mm) and 1.5 X TBE buffer in a cold room (4°C). Starting voltage was 250V till the samples entered in the gel, then the voltage was reduced to 200V and the gel was run for two hours at this voltage in a Hoefer Scientific Gel Apparatus. Under these conditions, the temperature of the buffer behind the plates was 10°C. The SSCP bands were visualized by silver staining technique. The mobility of SSCP bands in normal control DNA was compared with the mobility of the bands of resistant samples DNA.

RESULTS

Isoniazid (Isonicotinic acid hydrazide, 4-pyradinecarboxylic acid hydrazine) is highly active first line drug against the *M. tuberculosis* complex (*M. tuberculosis, M. bovis, M. africanum* and *M. microti*) (Youatt, 1969). It is a pro-drug that requires the activation of catalase-peroxidase enzyme (Zhang *et al.*, 1992). Soon after the INH was introduced in clinical treatment of tuberculosis in 1952, it was founded that clinical isolates frequently loose the catalase-peroxidase enzyme activities with the development of INH resistant. INH resistance is caused not only by the mutation of *Kat*G gene but other genes, including *inhA*, *Kas*A and genes under study also play a role in the resistance development of INH, but *Kat*G gene is present in the variable regions of the genome containing repeated DNA sequence which is a cause for the instability of this region and in turn contribute to the high frequency of *Kat*G gene mutation in INH resistant strains (Zhang & Young, 1994). So due to this high frequency of *Kat*G mutation in INH resistance this gene was selected in this study.

In this study a total of 31 *Mycobacterium tuberculosis* strain were found resistant to INH with or without the combination of other drugs on Lewenstein-Jensen (L.J) culture drug sensitivity medium. These 31 INH resistant strains were from 113 L.J culture medium positive strains. Out of these 31 INH resistant strains 24 were included to detect mutation in well reported *Kat*G gene and to know the effect of this mutation in resistance development. All the strains were also confirmed on molecular level (with PCR) and these were the members *of Mycobacterium tuberculosis*

Fig. 1. SSCP-PCR result of Isoniazid (INH) resistant cases (n=24)

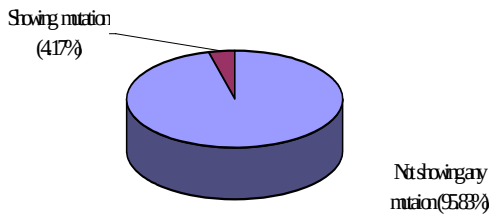
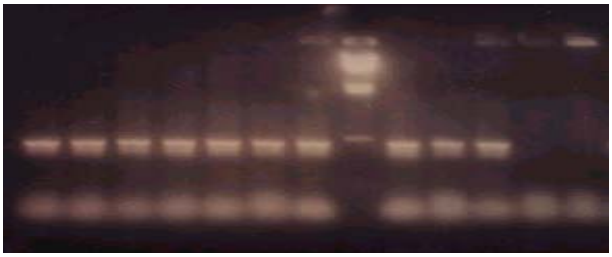
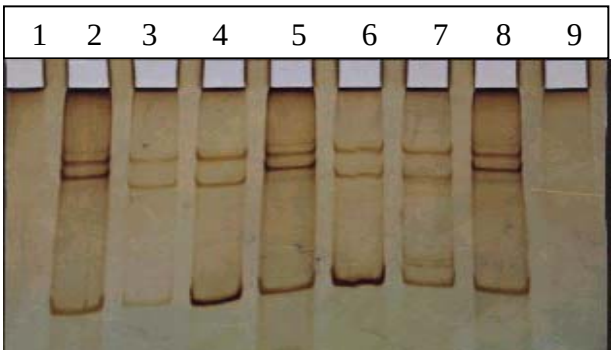


Fig. 2. PCR amplification of 541-bp fragment from IS986 gene of *Mycobacterium tuberculosis* complex



Lane 1,2,3,5,6,7,9,10	Samples of *Mycobacterium tuberculosis*
Lane 8	Molecular Weight Marker (λ DNA III digested)
Lane 12,13	Negative control
Lane 11	Positive control (541-bp)

Fig. 3. Detection of (228-bp) amplified fragment of *Kat*G gene on 20% PAGE



Lane 2,5,8	Wild type strains H37 Rv.
Lane 3,4,6,7	Same (INH) resistant strain run repeatedly.
Lane 1,9	Negative controls

complex as shown in Fig. 2. For the detection of these mutations many techniques were available including, DNA sequencing, dideoxy fingerprinting (ddF) and single strand confirmation polymorphism (SSCP) which could be used to detect INH mutation. Of these the first two techniques were either expensive or time consuming, where the last one was rapid and economical. So, SSCP method was selected for this study. When the amplified products of *Kat*G gene of INH resistant strains were detected on non-denaturing polyacrylamide gel electrophoresis (PAGE), out of 24 INH resistant strains only one (4.17%) had a detectable change in migration for ssDNA fragment. No suitable change in migration was observed for ssDNA fragment derived from 23 (95.83%) other INH resistant strains which were confirmed resistant to INH on L.J culture drug sensitivity medium as shown in Fig. 3 and the percentage is also depicted in Fig. 1.

DISCUSSION

A total of 31(27.43%) out of 113 culture positive strains were found resistant to INH with or without the combination of other anti-TB drugs on L.J medium culture. These findings were quite similar to the previous study conducted by Idrees *et al.* (1999) in which this ratio was 26.19%. Being an important drug in TB disease treatment the high rate of resistance against this drug was an alarming threat. This high rate in INH may be due to its common use in both initial and continuos phase or it's inadequate and sub optimal use in therapy as mentioned in our previous study (Iqbal *et al.*, 2001). So the proper use of this drug with the combination of other recommended anti-TB drugs should be made sure. With the importance of this drug we were hopeful to develop a rapid detection technique on molecular level for INH resistant cases and with this purpose the present study was designed.

For INH resistant strains the mutation in *Kat*G, *inh*A, *kas*A and *ahp*C genes has been previously reported, but some studies reveal that INH resistant strains have no mutation in these genes. These studies indicate that new undefined genes are also involved in INH resistance. It will be an interesting to see if following genes are identified. It will not only help to understand how INH works but will also facilitate to design a molecular assay for the more efficient detection of INH resistant organisms (Zhang & Amalio, 2000).

In our findings, out of these 24 strains only one (4.17) had a detectable change in migration for DNA fragments. No suitable change in migration was observed for ssDNA fragment derived from 23 (95.83%) INH resistant strains confirmed on L.J. drug sensitivity culture medium. The findings of present study indicates that mutation of *Kat*G gene of *Mycobacterium tuberculosis* is not reliable indicator of INH resistance, because many other genes including *inh*A, *kas*A, *ahp*C and genes under study may also be a cause of resistance against INH. So, these genes should also be taken under consideration for the detection of INH resistance. Similar problem was also reported previously (Van Doorn *et al.*, 2001) in which the mutation of *Kat*G gene of *M. tuberculosis* as a reliable indicator for INH resistance detection was rejected. In other studies, it has also been suggested that detection of INH resistance on molecular level is much more complex because it has also been reported that alteration in at least four genes *Kat*G, *inh*A, *kas*A, *ahp*C and many other genes is also involved in the resistance development of INH anti-TB drug (Zhang *et al.*, 1992; Wilson & Collins, 1996).

CONCLUSION

Regarding the role of *KatG* gene to INH resistance our findings have three implications: First, the percentage of isolates (30-60%) of the previous studies with INH resistance which is supposed to be mutation in *kat*G gene has been over estimated. Second, the changes in *Kat*G gene are not indicator of INH resistance of TB organism. Third, the development of SSCP-PCR or other diagnostic PCR technique for the detection of INH resistance should not be based only on the *Kat*G gene.

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