# katG (SER 315 THR) Gene Mutation in Isoniazid Resistant Mycobacterium tuberculosis

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**Citation**

Marahatta SB, Gautam S, Dhital S, Pote N, Jha AK, Mahato R, Mishra S, Poudel BH, Ramasoota P, Kaewkungwal J, Singhasivanon P. katG (SER 315 THR) Gene Mutation in isoniazid resistant Mycobacterium tuberculosis. *Kathmandu Univ Med J* 2011;33(1)19-23.

## ABSTRACT

**Background**

Isoniazid (INH) together with Rifampicin (RFP) forms the cornerstone of a short chemotherapy course for tuberculosis (TB) treatment. Mutation at codon 315 of *katG* gene is most prevalent in isoniazid resistant *Mycobacterium tuberculosis* (MTB) and is high in area with high TB incidence. Polymerase Chain Reaction Restriction Fragment Length Polymorphism (PCR-RFLP) has been found to be a reliable and effective tool for the identification of the specific gene alteration.

**Objective**

The objective of this study was to screen Ser315Thr mutation of *Kat*G gene of INH resistant MTB strain by PCR-RFLP technique.

**Methods**

Altogether 37 INH$^r$ MTB isolates obtained from German Nepal Tuberculosis Project (GENETUP) Kathmandu Nepal was included in the study. Deoxyribonucleic Acid (DNA) extraction was performed according to protocol of SORPOCLEAN™ from the culture isolates. Amplification of the fragment with *kat*G codon 315 was performed in a Biometra Thermocycler using primers. The amplified fragment was cleaved with *Msp*I. The restriction fragments obtained were electrophoresed in a 2% agarose gel and were visualized using transilluminator.

**Results**

The *kat*G Ser315Thr mutation was observed in 23 (62.2%) out of 37 INH resistant isolates. The drug susceptibility profile of INH$^r$ MTB isolates showed all isolates to be resistant to INH and RFP whereas 26 and 27 MTB isolates were resistant to Ethambutol (EMB) and Streptomycin (S) respectively. Seventeen (17) patients were harbouring *kat*G gene mutated strain among Ethambutol and Streptomycin resistant cases.

**Conclusion**

The study identified high prevalence of Ser315Thr mutation in *kat*G. The isolates harbouring this mutation were also simultaneously resistant to RFP. Ser315Th could be a potential genetic marker for predicting MDR-TB.

## KEY WORDS

*Isoniazid resistant MTB, katG gene, Mycobacterium tuberculosis, PCR-RFLP, mutation.*

## INTRODUCTION

Tuberculosis (TB) is the leading cause of mortality and accounts for 26% of all preventable adult deaths globally.[1] According to WHO, 8 million cases of TB occur each year, resulting in 3 million deaths. In addition overall one third of worlds' population is currently infected with the TB bacillus, 5-10% becoming sick at sometime during their lifetime. [2]

Currently TB is treated with an initial two month regimen comprising multiple antibiotics- isoniazid, rifampicin, pyrizinamide and ethambutol (HRZE) to ensure that the mutants resistant to even a single drug do not emerge. In next four months only isoniazid and rifampicin are administered to eliminate any persisting tubercle bacilli.[3] Isoniazid (INH) together with Rifampicin (RFP) forms the cornerstone of a short chemotherapy course for tuberculosis treatment. Mutation at codon 315 of *katG* gene is most prevalent in isoniazid resistant *Mycobacterium tuberculosis* (MTB) and is high in area with high TB

incidence. The prevalence of mutations at codon 315 varies greatly in different geographical regions.[4] The prevalence of the *kat*G (AGC ACC) mutation among MDR-MTB strains in the world, varies, being low in area with low TB incidence and high in area with high TB incidence.[5] China and India carry almost 50% of worlds MDR-TB burden.[6] Nepal, being geographically situated between these two nations with open border with India cannot be undermined for upcoming alarming situation of MDR-TB epidemics. Thus, detection of mutation in *katG* gene in Nepalese strains finds its rationale. The magnitude and trends in MDR tuberculosis are epidemiologically important to monitor, the estimation of the burden of disease is programmatically relevant in shaping policies for screening and treatment. The objective of this study was to screen Ser315Thr mutation of *Kat*G gene of INH resistant MTB strain by PCR-RFLP technique.

## METHODS

A total of 37 strains recovered from different adult patients (age range from 15 to 68 years) were studied. These patients originated either from Lalgadh hospital, Janakpur; Birjung Medical College, Birjung; GENETUP, Kathmandu and National Tuberculosis Centre, Bhaktapur. In Nepal, German Nepal Tuberculosis Project (GENETUP) is the national reference centre for drug susceptibility testing for mycobacteria, and receives all specimens detected in the country as a whole. Clinically and epidemiologically relevant information from each patient was obtained.

### DNA Extraction from MTB isolates

DNA extraction was done as guided by General Protocol for high DNA specimen of SORPOclean™. A loopful of each bacterial growth on Lowenstein Jensen medium was suspended in 1ml distilled water, warmed to room temperature (15-20°C). The 20µl proteinase K solution 200µl of sample and 200µl of lysis solution was added in 1.5 ml microcentrifuge tube. After vortexing (Taurus Scientifica) for 2-3 min. and incubation (56°C/10min), 230µl of 96% ethanol was added and mixed, and then the mixture was transferred into column. It was then centrifuged in a research centrifuge (REMI Instruments) at 6000g for 1min.Washing was done using Wash solution 1 and Wash solution 2 respectively with 500µl each. Finally 100µl of elution buffer pre-warmed at 70°C was added to elute purified DNA. The purity of DNA was determined by A260/A280 (optical density of DNA measured at 260 and 280 nm) using spectrophotometer (Thermoscientific).

### PCR procedure for katG gene amplification

Amplification of fragment with *kat*G codon 315 was performed in a Biometra Thermocycler with primers [Operon, Lot#1188685] *kat*G F (5'AGCTCGTATGGCACCGGAAC3') and *kat*G R (5'AACGGGTCCGGGATGGTG3') in a 25µl of a PCR mixture (1 U Taq DNA polymerase; 200µM each dNTP's; 1.5mM $Mgcl_2$; 15pmol of each primer; 2.5 µl of buffer [Fermentas, lot#00022114]; 1µl DNA sample) under the following condition (Initial denaturation at 95°C/10min; 30cycles of 94°C/1min; 55°C/1min ; 72°C/1min and a final elongation at 72°C/4min). Control reactions for detection of false positive results due to contamination with previously amplified amplicons were performed as follows: a negative control (distilled water) was included in each PCR run; no contamination was detected. A control sample (DNA extracted from $INH^S$ –H37Rv; Gene Bank accession no: X68081) was included in each PCR run; it showed clear band at 153bp

### Agarose gel electrophoresis

The amplicons were detected by 2% agarose gel electrophoresis stained with e ethediul bromide. MTB strain H37Rv and a PCR reaction without MTB DNA were used as positive and negative control in the PCR reaction respectively.

### Screening of mutation by RFLP

PCR amplicons were digested by *MspI* (Fermentas, lot no# ER 0541) incubating the mixture containing 10µl PCR product; 17.0µl NFW; 2µl 10x buffer tango (Fermentas, lot no# 00033251) and 1.0µl *Msp*I, at 37°C/3 hours. The mutation AGC (Ser) to ACC(Thr) creates an additional *Msp*I site (CCGG) and thus can be detected by use of this restriction endonuclease As a result, the longest RFLP product obtained was 132bp for $INH^r$ isolates with mutated 315 ACC allele and 153bp for *kat*G codon 315 wild type or differently mutated allele. The digested products were detected by 2% agarose gel electrophoresis stained with ethediul bromide. MTB strain H37Rv, wild type was used as a control in the digestion.

These indicative bands could be clearly discriminated in 2% agarose gel electrophoresis; they were the only visible bands, while 10 to 21 bp bands ran out of gel and were not considered. The electrophoresis was performed as described above.

### Data analysis

Data were analyzed manually as well as using SPSS Version16 and findings were interpreted according to frequency distribution and percentage. Data were presented in tables and figures.

Ethical approval for the study was taken from Institutional Review Board Kathmandu University School of Medical Sciences Dhulikhel Nepal.

## RESULTS

Using the *kat*G primers, amplicons were detected in 37 isolates. RFLP analysis of 37 *kat*G gene showed Ser315Thr mutation in 23 (62.2%) MTB strains. The RFLP pattern for isolates is shown in figure 1.

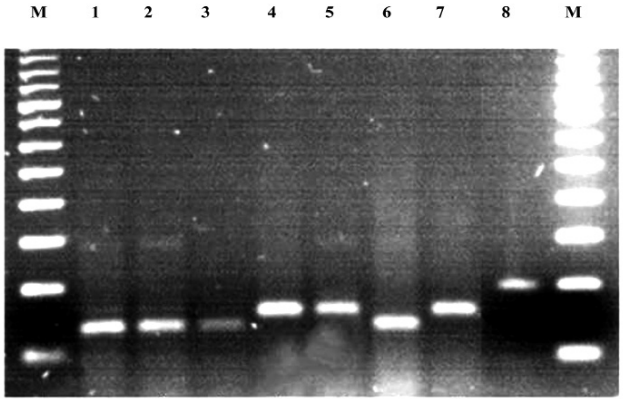


**Figure 1.Gel electrophoresis of amplified katG fragment and the products of its digestion**

The figure shows that the Lanes 1, 2, 3 and 6, are products obtained by *MspI* digestion of *kat*G gene of INH$^r$ strains with mutated *kat*G (315ACC); 4, 5 and 7, are products obtained by *Msp*I digestion of *kat*G strain with wild type or differently mutated strain; lane 8 is the undigested amplified 200bp *kat*G fragment; M represents the100 base pair DNA ladder (SORPO. Lot no# 00018146). A total of 37 randomly selected INH$^r$ isolates were examined.

**Table 1. Sex-wise distribution of mutation**

| Case | Male | Female | Total |
|---|---|---|---|
| Mutation | 18 | 5 | 23 |
| Wild Type or differently mutated | 7 | 7 | 14 |
| Total | 25 | 12 | 37 |

As per sex-wise distribution, the male patients outnumbered females. Among 25 male subjects 18 (72%) were harbouring mutated strain of MTB and 7(28%) were harbouring wild type or differently mutated strain. Among 12 female subjects 5 (41.6%) were harbouring mutated strain of MTB and 7(58.4%) were harbouring wild type or differently mutated strain as shown in table 1.

As per resistance pattern of other first line drugs among MDR-MTB isolates with *kat*G gene mutated and Wild type (WT) or differently mutated cases. 17 (73.9%) each were found to harbor cross-resistance with ethambutol and streptomycin.

As depicted in Table 2, the drug susceptibility profile of MDR-MTB isolates showed that poly-drug resistance pattern predominated with 62.1% being resistant to HRSE; 13.5% with HRS and 5.4% with HRE. However, no mono-drug resistant to either H or R was observed. The highest no. of *kat*G mutation was found in HRSE resistant strains.

**Table 2. Drug Susceptibility Profile of MDR- MTB isolate**

| Drug Resistant Profile | n | PCR-RFLP study | |
|---|---|---|---|
| | | Mutation | Wild Type or differently mutated |
| H | 0 | 0 | 0 |
| HR | 7 | 4 | 3 |
| HRE | 2 | 2 | 0 |
| HRS | 5 | 2 | 3 |
| HRSE | 23 | 15 | 8 |
| Total | 37 | 23 | 14 |

H: Isoniazid; R: Rifampicin; E: Ethambutol; S: Streptomycin.

**Table 3. Resistance Pattern of 1st line Drugs**

| Drugs | Isoniazide | Rifampicin | Ethambutol | Streptomycin |
|---|---|---|---|---|
| **Patient with mutated strain** | 23 | 23 | 17 | 17 |

## DISCUSSION

Multidrug resistant tuberculosis is entirely a man made phenomenon[3] but not due to emergence of novel resistance mechanism[7]; whereby MTB bacilli acquire resistant to at least isoniazid and rifampicin.[3] The ability of DOTS programs to reduce transmission and incidence of both drug susceptible and drug- resistant tuberculosis is debatable; while some studies have shown successful reduction of drug resistance under WHO strategy, others have demonstrated an "amplifier effect" of increasing drug resistance under DOTS- prescribed short course therapy.[8]

The most frequent mutation patterns of isoniazid, rifampicin and streptomycin resistant strain occurred at codon 315 (55-90%) of *kat*G; codon 531 (40-60%) and codon526 (10-30%) within RPF resistance determining region of *rpo*B; and codon 43 (47-79%) of *rps*L within Streptomycin resistant MTB. Mutation in *kat*G gene occurs in a 100 fold higher frequency than *rpo*B gene [9] and is regarded as first step in evolution of MDR-MTB [10- 13] and also creates more possibility for acquiring mutations in other genes.[13-15] The combination of INH resistance and maintained virulence might make it possible for some INH resistant strains, especially those with the *kat*G Ser315Thr mutation, to acquire extra drug resistance and become MDR-TB. [13] The molecular basis of resistance to isoniazid is more complex and is caused by a variety of mutations in four different genes of MTB *i.e*. *kat*G encoding catalase peroxidase, *inhA* encoding the enoyl acyl carrier protein (ACP) reductase, *kas*A encoding β-ketoacyl ACP synthase and *ahp*C encoding alkyl-hydroperoxide reductase e.t.c. Even then, nearly 5-10% of isoniazid-resistant *M. tuberculosis* isolates do not have an identifiable mutation.[16]

This study was conducted to gain further insight into molecular basis of INH$^r$ MTB circulating in Central development region of Nepal. We found that mutation in *kat*G gene (Ser315Thr substitution) was responsible for 62.2% of INH$^r$ strains. We compared our result of mutation at *kat*G codon 315 associated with INH resistance and results of demographic findings with those of earlier studies done worldwide.

The prevalence of *kat*G S315T substitution in MTB strains vary all over the world especially with regard to TB prevalence.[17-19] In place like Singapore [20] and Madrid [21] where prevalence of TB incidence is intermediate and low, mutation has been reported in 26-30% isolates. In contrast the Ser315Thr mutation accounted for INH resistance in 52-64% of strains in Central Africa.[22-23] Several studies have revealed that mutation in *kat*G gene is responsible for 60-70% of INH$^r$ strains.[24] A study by Negi S.S *et al.,* [25] in 2006, in India revealed *kat*G S315T mutation in 74.19% of strains of MTB from Delhi. In Nepal where incidence of all forms of TB is 173 cases per 1, 00,000 population[3] the Ser315Thr mutation we found was not as high as those of north-western Russia (93.6%)[26] Germany (86.41%)[28] , Lithuania (85.71%) [15] but was concordance with other results in Mexico (67.61%), Poland (66.3%), and Australia (65.4%).[29] Similarly, Wang Y.C *et al.,* [18] from China reported 68.6%; Hass W.H [30] from West Africa reported 60% of INH$^r$ strains associated with mutation in *kat*G due to S315T substitution. Our finding of mutation in the *kat*G Ser315Thr substitution (*i.e* 62.2%) is similar to those reported in other parts of the world, which reflects a global pattern.

Furthermore, in current study association was conformed between the common *kat*G Ser315Thr mutation in INH resistant strains and the presence of resistance to other first line drugs (i.e. RPF, EMB, and S). These observations might suggest that the INH resistant with *kat*GS315Thr mutation may be more likely to develop resistance to other first line drugs. Hu Yi *et al.,* [13] in their study also found similar correlation between INH resistance and development of resistance to other first line drugs. Since all the INH$^r$ isolates were resistant to RPF as well so we concluded that all the strains under study are MDR-MTB isolates.

The present study identified *kat*G Ser315Thr mutation in Nepalese INH$^r$ isolates, so the results we obtained could not be compared with other local investigations. But extensive transmission of INH$^r$ MTB was observed in Eastern region of neighbouring country, China. Most INH$^r$ isolates have strong transmissibility, full virulence and survival advantage even under the drug pressure in the host. [13] The study conducted in China presented mutation with 68.6% [18] cases among INH$^r$ MTB strains; India, the next neighbouring country of Nepal presented mutation with 64.3 % [30] cases among INH$^r$ MTB strains. The samples we included were also included from two different areas of eastern terai which has open border with India. Thus, the chance, that Nepal may carry extensive transmission rate of INH$^r$-TB with *kat*G gene mutation cannot be ignored. On the other hand the increased possibility that the INH$^r$ MTB with the *kat*G Ser315Thr mutation in the index person is more likely to experience a series of subsequent mutations, leading to the accumulation of MDR and/ or extreme drug resistant(XDR)-TB.[31]

Furthermore, in current study we found number of male patients outnumbering the number of female patients with INH resistant isolates. In a molecular epidemiological study of Hu Yi *et al.,*[13] in eastern China similar type of finding was stated where male outnumbered female patients with average age being 30 years. Similarly in his study, Pandey R.P [32] in Doti district of Western region of Nepal also found male patients outnumbering female patients with average age of male being 36.7 and female being 33.7 suggesting that the prevalence of MDR-TB within the country may vary among different age groups.

Early detection of the patients carrying INH resistant strains would facilitate the modification of treatment regimens and appropriate infection control measures can be taken in time to reduce the risk of further development and transmission of MDR-TB. An understanding on the molecular level of the mechanism of drug resistance in MTB will enable us to develop improved tools. It deserves further investigation to determine which mechanism may play the critical role in the epidemic of MDR-TB, since the implication behind it could be meaningful to evaluate the performance of local TB control as well as to determine the MDR-TB strategies.

## CONCLUSION

The study identified high prevalence of Ser315Thr mutation in *kat*G. The isolates harbouring this mutation were also simultaneously resistant to RFP. Ser315Th could be a potential genetic marker for predicting MDR-TB. The molecular detection of this mutation by PCR-RFLP in TB control programmes could help in early detection, management and containment of MDR-TB cases.

## ACKNOWLEDGEMENT

UNDP/World Bank/WHO Special program for Research and Training in Tropical Disease (TDR) Geneva Switzerland; Nobel College Kathmandu and GENETUP Kathmandu Nepal

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