Journal of Cardiovascular Disease Research
ISSN: 0975-3583, 0976-2833 VOL 15, ISSUE 11, 2024

Original Research Article

Pattern of Isoniazid Monoresistance among Pulmonary Tuberculosis Patients at Tertiary Care Centre

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ABSTRACT

Introduction: Tuberculosis is an infectious disease, which remains killer disease of all times. Drug resistance in the first line drugs makes the situation worse. Major obstacle in the management of tuberculosis is emergence of drug resistance. The molecular line-probe assay provides rapid diagnosis and early management of MDR-TB. It detects mutations of *katG* and *inhA* genes associated with isoniazid (INH) resistance in *Mycobacterium tuberculosis* isolates.

Aim and Objectives: To detect isoniazid mono resistance and determine drug resistance pattern of isoniazid in specimens of patients having pulmonary tuberculosis by Line Probe Assay.

Material and Methods: The retrospective study was conducted in Tb culture and DST laboratory, department of Microbiology for 18 months period from January 2021 to June 2022. Samples were further proceeding for sputum decontamination, DNA extraction followed by amplification and hybridization.

Results: Total 9619 samples received during the study period and out of which 6048 samples were pulmonary specimens and 3571 were extra pulmonary specimens. Out of 6048, pulmonary TB was detected in 4900. Out of 4900, Isoniazid mono resistance was in 360 samples. Isoniazid mono resistances were 360(7.3%). In which, KatG mutation was present in 226(62.7%), InhA in 114(31.7%), both KatG gene and InhA gene were present in 11(3.1%) and 9 (2.5%) were Inferred.

Conclusion: It concludes that Isoniazid is the most important drug for TB treatment and resistance to it can lead to failure of TB management. With the help of line probe assay, we can diagnose TB and drug resistance in a single day and this will allow us to start appropriate treatment.

Keywords: Pulmonary tuberculosis, Isoniazid, Kat G gene mutation, InhA gene mutation

INTRODUCTION

Tuberculosis (TB) is the ancient disease and remains the greatest killer disease of all times (1). The explosive emergence of drug resistance has worsened the situation and paralyzing the regimen applicability of potent first line anti TB drug like isoniazid and rifampicin (2). The emergence of MDR and XDR, clinicians have with hardly any treatment options for TB, and tuberculosis spread has already created a serious threat to public health. Inappropriate drug regimen, patient defaulting, previous anti-TB treatment, delay in diagnosis and initiation of effective treatment, and primary infection with MDR TB strains are responsible for MDR and XDR TB (3). First line anti TB drug isoniazid (INH) is a prodrug that diffuses passively into growing bacilli. The catalase-peroxidase (KatG) enzyme, encoded by the *katG* gene, converts INH to the active form and then act by inhibiting mycolic acid biosynthesis, which is an essential component of the mycobacterial cell wall (4). The InhA or enoyl-acyl carrier protein (ACP) reductase enzyme is encoded by the *inhA* gene, which is involved in mycolic acid biosynthesis. Activated INH binds to and inhibits this enzyme (5). Because of high early Bactericidal activity combined with a good safety profile of isoniazid, it is considered as a backbone therapy in treatment tuberculosis (6). But INH mono-resistance increases the likelihood of negative Treatment outcome and progression to MDR TB (7,8,9).The molecular basis of INH resistance is mutations in the *katG*, *inhA*, or *inhA* promoter genes of *M.tb*, to which about 80% of its phenotypic resistance in the world is attributed (10).

Line Probe Assay (LPA) is designed to identify *M. tuberculosis* complex and simultaneously detects mutations related to drug resistance. LPA has high sensitivity and specificity for detection of both isoniazid and rifampicin resistance. LPA uses PCR (polymerase chain reaction) and reverse hybridization methods for detection of mutations (11). Two types of LPA, GenoType®MTBDRplus (for detection of INH and RIF resistance), and GenoType®MTBDRsl (for detection of resistance against ethambutol, fluoroquinolones and aminoglycosides) (Hain Lifescience, Nehren, Germany) are commercially available that are being used to detect drug resistance genes of MTB. In 2008, genotype MTBDRplus LPA has been recommended for rapid detection of drug resistance of MTB by WHO (12).

MATERIAL AND METHODS

The retrospective study was conducted in Tb culture and DST laboratory, department of Microbiology for 18 months period from January 2021 to June 2022.

Sputum samples of pulmonary tuberculosis patients were received in TB culture and DST laboratory, department of Microbiology. They were processed for isolation of mycobacterium tuberculosis and drug resistance pattern for isoniazid mono resistance by Line Probe Assay.

Total 9619 samples received during the study period and out of which 6048 samples were pulmonary specimens and 3571 were extra pulmonary specimens. Out of 6048, pulmonary TB was detected in 4900. Samples were further processed for sputum decontamination, DNA extraction followed by amplification and hybridization.

Sputum specimens are viscous material contaminated with normal flora so Processing involves pre-treatment of the sputum specimen. So digestion was done to free the TB bacilli from the mucus, cells or tissue in which they may be embedded and decontamination was done by N-acetyl L-cysteine/NAOH in BSC 3 to eradicate normal flora that grow more rapidly than TB. All steps have been performed in a BSC at BSL3 Laboratory with using appropriate BSL3 PPE. A written checklist of materials and tools needed and a worksheet with list of specimens (identifiers) to be tested was prepared. The BSC was decontaminated with freshly diluted 1.5% bleach (20 min), followed by 70% alcohol. The working area was covered with paper towels and disinfected. A discard container with a plastic bag and freshly diluted 1.5% bleach was used.

DNA EXTRACTION: It was done in BSL 3 lab. Pellet bacteria by centrifuging specimen on table top centrifuge with an aerosol tight rotor in BSC for 15 min at 10000 x g. DNA Extraction was done with Genolys by adding 100 µL lysis buffer to the sediment and incubated the tube in water bath at 95 ºc for 20 minutes and then added 100µL of neutralization buffer and tube vortexed and centrifuge the specimen for 5 minutes at 13000 x g. and transferred supernatant containing DNA to a new 1.5ml screw cap vial. DNA extraction from Lowenstein Jensen culture media, 1µl sterile disposable inoculation loop was used to collect bacteria from media and incubated for 20 min at 95°C in the water bath. Rest of the procedure was same as the above.

MASTER MIX PREPARATION: PCR tubes was labeled in line with numbers of specimens and controls and then dispensed 45 ml master mix into each PCR tube, 5 ml of either AM-A or AM-B was added to prepare master mix into negative control PCR tube.

	Time and Temperature	**Culture samples**	**Direct patient material**
Denaturation	15 min 95°C	1 cycle	1 cycle
Denaturation	30 sec 95°C 2 min 65°C	10 cycles	20 cycles
Denaturation Annealing Elongation	25 sec 95°C 40 sec 50°C 40 sec 70°C	20 cycles	30 cycles
Final Extension	8 min 70°C	1 cycle	1 cycle

AMPLIFICATION: The amplification was done in Thermocycler. Amplified product was stored at -20°C.

Table 1: Amplification Profile

DENATURATION AND HYBRIDIZATION: Hybridization was done in pre warm TwinCubator at 45°C. Pre-warm HYB and STR solutions (green and red) were kept at 45°C in water bath for 15 minutes total. 20 µl DEN (denaturing solution) was added to each well and then 20 µl of corresponding amplified DNA sample to each well, mixed well by pipetting up and down and incubated for 5 minutes at room temperature. DNA strips from tube were removed. 1 ml HYB (hybridization solution) was added to each well and gently shaked to homogenize solution. After that, 1 strip was added to each well with colored marker facing up. Tray was placed on TwinCubator for 30 minutes at 45°. HYB poured off by sterile pipette into a discard container containing undiluted bleach solution. Then 1 ml STR (stringent buffer) added per well and incubated for 15 minutes in TwinCubator at 45°C. Completely removed STR as previously described for HYB removal. Prepared diluted Conjugate and Substrate in 15 ml conical vials by diluting 1:100 with corresponding Con-D and Sub-D. Added 1 ml RIN (rinse solution) and incubated for 1 minute. After removing RIN, 1 ml of diluted Conjugate was added per well and incubated for 30 minutes. After removing solution and 3 times washed for 1 minute with 1 ml RIN. Remove RIN and washed with 1 ml distilled water. Then 1 ml of diluted substrate was added. Placed on TwinCubator under aluminum foil for a maximum 10 minutes. Washed twice for 1 minute each with distilled water. Then with forceps, strips were transferred to the GenoType MTBDRplus Results Sheet provided with the kit.

INTERPRITATION OF RESULTS: Read results by lining strips up to code provided with kit.

RESULTS

Out of 4900 samples tested, 4388 samples were sensitive to both drugs (Isoniazid and Rifampicin) and 562 samples were resistant to either one drug or both drugs (Isoniazid and Rifampicin). As shown in table 2 Sensitivity rate was 88.5% and resistant rate was 11.5%.

Table 2: Sensitivity and resistance rate for first line drugs of pulmonary tuberculosis (in %)

TOTAL SAMPLES TESTED	FL LPA SENSITIVE n (%)	FL LPA RESISTANT n (%)
4900	4338 (88.5%)	562 (11.5%)

4900 First line LPA were done, out of which, Isoniazid Mono resistance were 360(7.3%). Figure 1 shows isoniazid mono resistance.

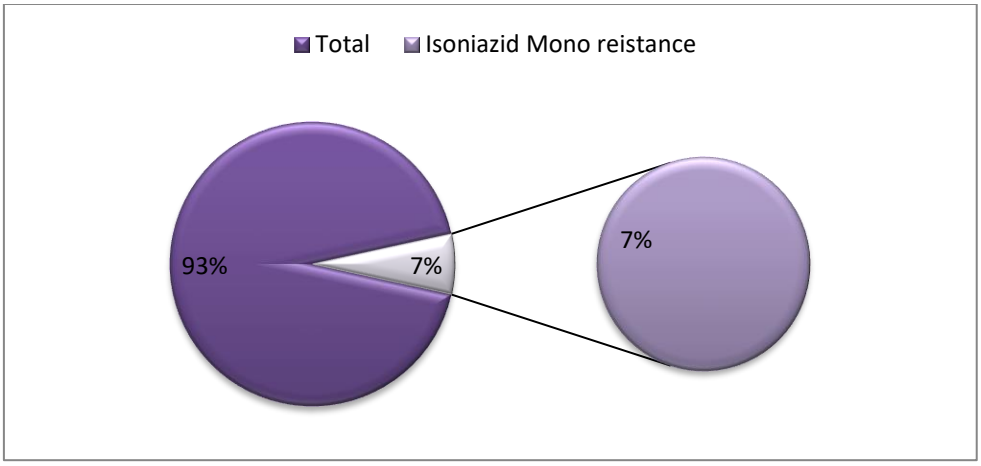

Figure 1: Distribution of Isoniazid Mono resistant cases among Pulmonary TB patients

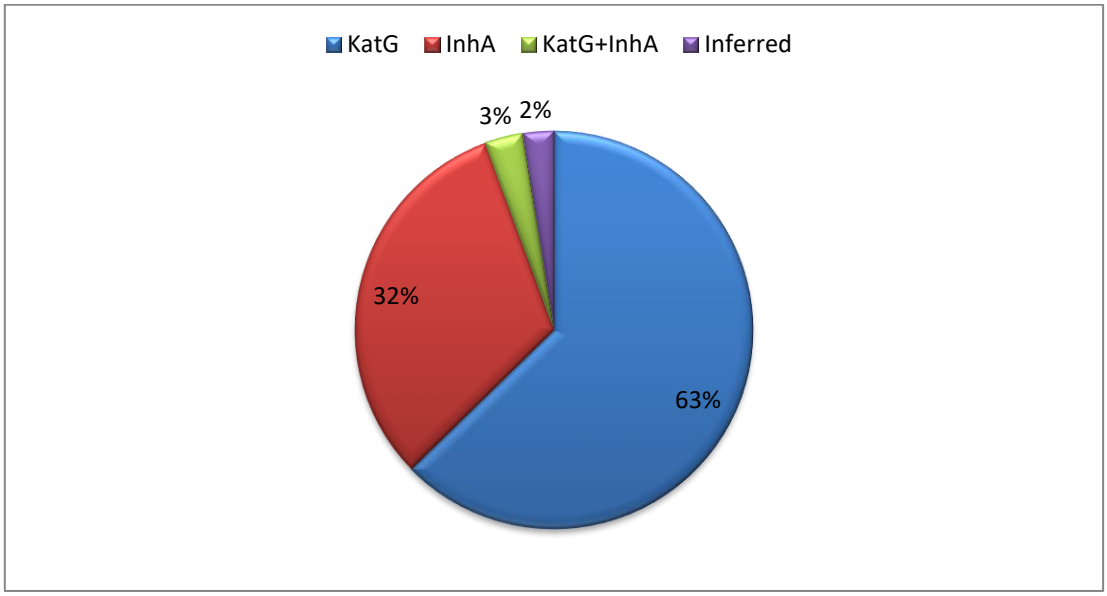

Figure 2: Pattern of Gene mutation in INH Monoresistant mycobacterium TB strains

Isoniazid mono resistance were 360(7.3%). In which, KatG mutation was present in 226(62.7%), InhA in 114(31.7%), both KatG gene and InhA gene were present in 11(3.1%) and 9 (2.5%) were Inferred. Figure 2 shows gene mutation pattern.

Table 3: Gender wise distribution of isoniazid mono resistance in pulmonary tuberculosis

Gender	Number	Percentage
Male	259	71.9
Female	101	28.1
Total	360	100

As shown in table 3, out of 360, Isoniazid mono resistance, 259 (71.9%) were males and 101(28.1%) were females. Male to Female ratio was 2.6:1.

Out of 360 isoniazid mono resistance, the majority of the patients were between the age 30-44(36.7%) years followed by the age 15-29(26.7%). Figure 3 shows age wise distribution of isoniazid resistance.

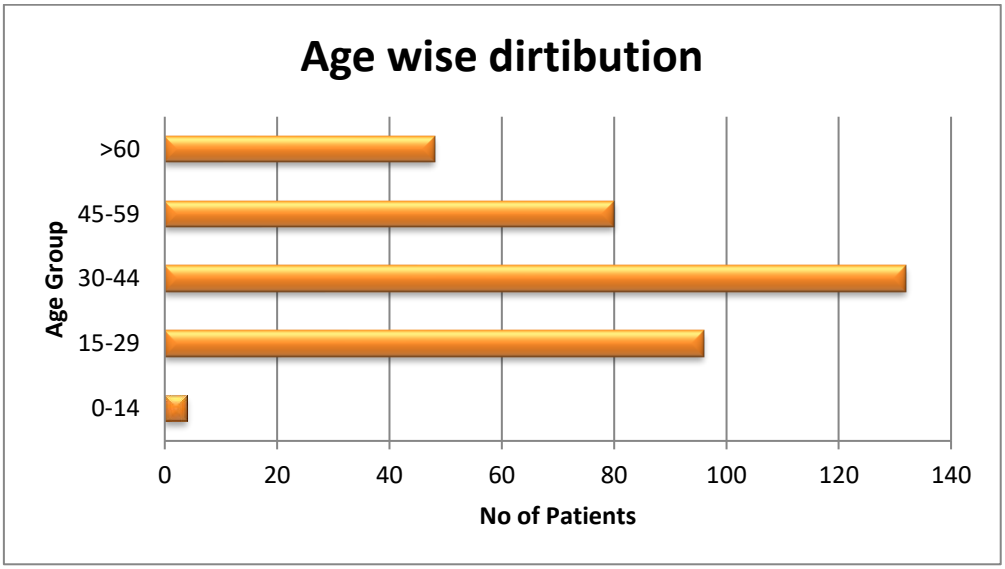

Figure 3: Age wise distribution of Isoniazid mono resistance

DISCUSSION

Tuberculosis is an important cause of death worldwide and public health crisis even though it is preventable, treatable and curable. Drug resistance in tuberculosis is a major public health challenge in developing countries. Due to emerging of multi drug resistance, Mycobacterium tuberculosis strains have become a major obstacle in the management of TB (13). *M. tuberculosis* can acquire antibiotic resistance via chromosomal mutations but not through horizontal transfer of genes (14). Mutations occur mainly in single nucleotide polymorphisms insertion and deletions in genes encoding drug targets or drug converting enzymes (15).

In this study, total 4900 valid LPA were done in which 4388(88.5%) were sensitive to all first line drugs and 562(11.5%) were resistant either mono drug resistant or multi drug resistant. Study of Kerala by Murthy N.S. et al. (16) showed 85.1% drug susceptibility and 14.9% drug resistant TB. While, 68.76% drug susceptibility and 31.24% drug resistant detected in Andhra Pradesh Study by Prashad P.G. et al (17).

This might be due to Geographical, sample size and strain variation. Drug resistance also depends on Attitude of patients, co-morbidities of patients, long-term treatment follow up and health education of patients (16).

Total 4900 pulmonary patients LPA were done in present study, out of which 360(7.3%) Isoniazid mono resistance was detected which is comparable to study conducted by Singhal R. et al. (18) in Punjab, Murthy N.S. et al. (16) in Kerala, Vanzara S et al. (19) in Bhavnagar and Rashid O et al.(20) reported 7.1%, 6.5%, 6.8% and 6.28% Isoniazid mono resistance respectively.

Increasing Isoniazid mono resistance might be due to its broad therapeutic range makes it possible to achieve high drug concentration in tuberculosis lesion and they respond to conventional combination therapy without being recognized (21).

From 360 Isoniazid mono resistance, KatG mutations were found in 226(62.7%). Almost similar results were found by Bollela V R et al. (22) (54.5%), Pisto L et al. (23) (63.9%), and Bakhtiyaniriya P et al. (24) (66.7%). Study of Rajasthan by Charan A.S. et al. (13) also reported 65.1% isoniazid mono resistance.

InhA mutation in Isoniazid mono resistance was 30%. Charan A.S. et al. (13) reported 28.1% and Bakhtiyaniriya P et al. (24) reported 33.3%. while Pisto L et al. (23) and Bollela V R et al. (22) have found 13.4% and 40.9% InhA gene mutation respectively.

In current study, combined mutations of KatG and InhA were seen in 4.7% which is in line with study of Charan A.S. et al. (13), Prashad P.G. et al. (17) and Singhal R. et al. (18) observed 6.7%, 3.4% and 5.2% in Rajasthan, Andhra Pradesh and Punjab region respectively. While Pisto L et al. (23) was found 22.7% of combined mutation, which is higher than present study.

Combines mutation signifies higher degree of resistance to Isoniazid and high dose of INH is ineffective for patients (13) and therefore need to be considered for an individualized TB regimen (23).

In present study, Male to Female ratio was found to be 2.6:1 in which 259(71.9%) were males and 101(28.1%) were females. Study conducted by Murthy N.S. et al. (16) in Kerala shows 3.4:1 Male to Female ratio. Study conducted in Rajasthan by Charan A.S. et al. (13) reported 65.2% and Pisto L et al. (23) reported 58.4% in males respectively.

The mean age was 39 years of 360 patients, which is similar to study done by Bakhtiyaniriya P et al. (24) (mean age - 39 years) and Pisto L et al. (23) mean age - 37 years). Study conducted in Rajasthan by Charan A.S. et al. (13) and by Prashad P.G. et al (17) in Andhra Pradesh shows mean age 40.27±13.82 years and 41.5 years respectively.

CONCLUSION

The present study ascertained the prevalence of isoniazid mono resistance among TB isolates and pattern of gene mutation among resistant isolates. It concludes that Isoniazid is the most important drug for TB treatment and resistance to this drug can lead to failure of TB programs. Drug resistance should be suspected in patients not improving on standard drug TB regimen.

Isoniazid mutations are very closely related to high and low degree resistance to INH and therapeutic regimen is differ for two, it is important to know about gene pattern of patients.

By line probe assay, diagnosis and drug resistance in TB can be detected in a single day and this will allow starting appropriate treatment. Having an adequate knowledge on the molecular mechanisms of drug resistance in *M. tuberculosis* may be helpful in exploring new targets for drug development.

Surveillance or epidemiological studies should be done further to control spread of infection. Continued Surveillance through traditional culture and DST methods will remain important to individualize and customize treatment regimen for drug resistant TB.

Clinicians know how to interpret the results and are aware of the treatment implications of TB associated with the various INH resistance-conferring mutations. This knowledge of genetic mutations associated with INH resistance and effective implementation of new regimens could help to improve the effectiveness of MDR-TB treatment and thus improve morbidity and reduce further transmission.

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