Original Article

Correlation of *inhA* mutations and ethionamide susceptibility: Experience from national reference center for tuberculosis

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ABSTRACT

Background: Detection of ethionamide (ETH) resistance is crucial as it is part of antitubercular regime. It is crucial to examine the role of *inhA* gene mutations as a surrogate marker for the detection of ETH resistance, in the Indian context. The present retrospective study was designed with this objective. **Subjects and Methods:** The study was conducted in National Reference Laboratory within the tertiary care institute from January 1, 2018, to June 30, 2019, over 18 months duration. A total of 6612 sputum samples from presumptive multidrug-resistant tuberculosis (TB) patients were received from four districts of Delhi, outdoor and inpatients. Line probe assay (LPA) was performed for smear-positive or culture-positive samples for *Mycobacterium tuberculosis*. All isolates found to be INH resistant by LPA were cultured and phenotypic susceptibility to ETH was conducted for selected isolates as per the guidelines. **Results:** A total of 246 isolates were analyzed, for which phenotypic susceptibility to ETH and mutations in *inhA* were available. ETH resistance was detected among 87/108 (80.5%) isolates with *inhA* mutation. Sensitivity and specificity of *inhA* mutation for detection of ETH resistance were 80.5% and 83.8%, respectively. No *inhA* mutation was detected in 29/116 (25%) ETH-resistant isolates in our study, whereas ETH was found to be phenotypically susceptible in spite of the presence of *inhA* mutation among 21/130 (16.1%) isolates. **Conclusions:** Mutations in *inhA* gene in LPA predict ETH resistance with fairly good sensitivity and specificity. However, it is imperative to perform phenotypic detection of ETH resistance at proper concentration, in addition to detecting *inhA* mutation.

KEY WORDS: Ethionamide resistance, *inhA,* mutation, tuberculosis

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Submitted: 17-Feb-2021 **Revised:** 12-Apr-2021 **Accepted:** 24-Jun-2021 **Published:** 26-Oct-2021

INTRODUCTION

Tuberculosis (TB) remains one of the most challenging global health problems, especially as resistance to antimycobacterial drugs continues to rise in many countries worldwide. Isoniazid (INH), rifampin (RIF), pyrazinamide, ethambutol, or streptomycin (S) are "first-line" anti-TB drugs given to new or drug-sensitive TB patients.

Access this article online

Quick Response Code:

Website: www.lungindia.com

DOI: 10.4103/lungindia.lungindia_120_21

How to cite this article: Sarin R, Bhalla M, Kumar G, Singh A, Myneedu VP, Singhal R. Correlation of *inhA* mutations and ethionamide susceptibility: Experience from national reference center for tuberculosis. Lung India 2021;38:520-3.

Multidrug-resistant TB (MDR-TB), defined as resistant to at least RIF and INH, is becoming a catastrophic problem. There were almost 484,000 (range: 417,000–556,000) incident cases of MDR/RR-TB in 2018, of which 78% had MDR-TB and about 214,000 (range: 133,000–295,000) deaths from MDR/RR-TB.[1] In this scenario, the selection of "second-line" drugs is necessary to replace ineffective first-line drugs. The second-line drugs include fluoroquinolone (FQ), aminoglycosides (AG), capreomycin, ethionamide (ETH), prothionamide, cycloserine, linezolid, clofazimine, bedaquiline, and delamanid.

The World Health Organization has approved molecular tests, such as Xpert and line probe assay (LPA) as primary diagnostics due to their short turn-around time and accuracy. As per the Revised National Tuberculosis Control Program (RNTCP), all samples diagnosed as RIF resistant by Xpert or LPA are eligible for a shorter MDR-TB regimen of 9–11 months provided they meet the inclusion criterion.[2]

First-line LPA, besides detecting resistance to RIF by *rpoB* gene, also has *katG* and *inhA* genes for detection of INH resistance. Mutations within *katG* gene and *inhA* gene are associated with high-level and low-level resistance to INH, respectively.[3]

INH on activation binds to the enzyme, Nicotinamide adenine dinucleotide-dependent enoyl-ACP reductase encoded by *inhA* gene and inhibits mycolic acid, a crucial component of the mycobacterial cell wall.[4-6] ETH, an efficacious second-line drug, is used in a shorter-MDR-TB regimen.[2] It is a structural analog of INH and when activated inhibits *inhA* enzyme, thus causing inhibition of mycolic acid biosynthesis.[7] Thus, mutations in the promoter and structural regions of *inhA* gene confer co-resistance to INH and ETH.[6-8] Mutations in *inhA* hence have been considered as a surrogate marker for the early detection of ETH resistance. However, there are limited scientific data of *inhA* mutation and ETH resistance correlation from the Indian subcontinent.

The present retrospective study has been designed to ascertain the relevance of using *inhA* mutations in GenoType MTBDRplus as the sole marker of ETH resistance.

SUBJECTS AND METHODS

This retrospective study was carried out in the National Reference Laboratory within the Department of Microbiology, which is accredited for phenotypic and genotypic tests for TB as per the ISO 15189. The NRL is enrolled with Supranational Laboratory (Antwerp Belgium) for regular proficiency tested for *M. tuberculosis* drug susceptibility. During the study period, from January 1, 2018, to June 30, 2019, data of isolates with available *inhA* mutations and phenotypic ETH susceptibility was collected. A total of 6612 sputum samples from presumptive MDR-TB patients were received from four districts of Delhi under RNTCP, outdoor and ward patients in the study period, which were subjected to relevant tests.

These samples were subjected to and screened for the presence of acid-fast bacilli by Ziehl–Neelsen staining and processed by N-acetyl-L cysteine-Sodium hydroxide method of digestion and decontamination as per the standard protocol.[9] All smear-positive samples and culture positive for MTB were subjected to Hain Lifescience GmbH, Nehren, Germany based on PMDT guidelines.[2,10] Procedures for DNA extraction, master-mix preparation, multiplex amplification with biotinylated primers, and DNA hybridization were done after a thorough cleaning in dedicated rooms as per the manufacturer's instructions.[10] Interpretation of LPA strips and recording of mutations were done by a senior microbiologist. Any doubtful results were repeated. Any samples with RIF and/or INH resistance and additional resistance to FQ and/or AG/CP were considered for culture and susceptibility testing to ETH and other second-line antibiotics by MGIT960. Tubes with positive alerts were identified for the presence of *M. tuberculosis* by smear microscopy for serpentine cording and rapid immune-chromatographic test. Cultures positive for *M. tuberculosis* were subjected to DST ETH (5.0 µg/ml) using MGIT960 using a standard protocol as per the manufacturer's instructions.[11] During the study period, all samples were presented as frequency tables, and the proportion was calculated as required. Statistical parameters of sensitivity, specificity, positive and negative predictive value, and overall concordance were calculated using a 2 × 2 table. Study was waivered for taking consent from patients, as this is retrospective study.

RESULTS

A total of 246 isolates with available phenotypic susceptibility to ETH and mutations in *inhA* were analyzed.

In the present study, 87/108 (80.5%) isolates with *inhA* mutation showed ETH resistance and 109/130 (83.8%) isolates with absent *inhA* mutation were sensitive to ETH. Overall, a statistically 79.6% correlation was found between ETH resistance and *inhA* mutation [Table 1]. The sensitivity and specificity of *inhA* mutation for the detection of ETH resistance were 80.5% and 83.8%, respectively. Positive and negative predictive value was 75% and 83.8%, respectively

Table 1: Correlation between ethionamide susceptibility and inhA mutation

Mutation in inhA promoter region	Phenotypic susceptibility		Total
	ETH resistance	ETH sensitive	
inhA mutation present	87	21	108
inhA mutation absent	29	109	138
Total	116	130	246

ETH: Ethionamide

Among 116 ETH phenotypically resistant isolates, mutations in the promoter region of *inhA* were seen in 87/116 (75%) isolates. Mutations in − 15/−16 promoter region were detected in 72/87 (82.7%) of which 71 were C15T. Eleven mutations were in − 8 promoter region of *inhA* in which 8/11 (72.7%) and 2/11 (18.1%) had specific mutations T8C and T8A, respectively. Possible heteroresistance was seen in 4 isolates with additional C15T mutation. Among 29/116 (25%) ETH-resistant isolates in our study, no *inhA* mutation was seen, whereas among 21/130 (16.1%) isolates with *inhA* mutation, ETH was found to be sensitive.

DISCUSSION

ETH, like INH, is a prodrug activated by the ethA-encoded monooxygenase.[12-14] *M. tuberculosis* treated with ETH loses its acid fastness and its ability to synthesize mycolic acids. These are Group C, oral second-line anti-TB agents. ETH is used in the intensive phase in shorter MDR-TB treatment[15] as per the PMDT guidelines.[2] Hence, for early decision in all such cases, it is imperative to have a rapid sensitive and specific susceptibility testing methodology for ETH. Molecular methods such as LPAs are rapid and have higher throughput than MGIT960 or solid DST. LPA provides additional relevant information, which can help clinicians initiate suitable treatment. The level of resistance for INH can be determined using mutations in *katG* (high-level) and *inhA* (low level). Mutations in *inhA* promoter region also contribute to cross-resistance to ETH.[16]

In our study, 80.5% of isolates with *inhA* mutation had ETH resistance. In an Indian study from Mumbai, 95% of strains with *inhA* promoter mutation were associated with phenotypic ETH resistance.[17] In a study from Brazil, all strains with *inhA* mutation were resistant to ETH, while 94.1% of ETH resistant were found to have *inhA* mutations.[8] Lee *et al*.[18] from Korea studied 12 ETH-resistant strains and all had *inhA* mutations.[18] Brossier *et al*.[19] from France reported 62% mutations in *inhA* gene among ETH-resistant clinical isolates.[19]Muller *et al*.[20] from South Africa have reported c-15t mutation in the promoter region of *inhA* among 55% of ETH-resistant clinical isolates.[20] The above studies substantiate the significant association of *inhA* mutations and phenotypic resistance to ETH and INH. A wide range of correlations in various studies could be due to geographical variations in mutations in genes and varying sample sizes.

Among 29/116 (25%) ETH-resistant isolates in our study, no *inhA* mutation was seen. The plausible explanation is the presence of an alternative mechanism that could be responsible for conferring ETH resistance. Vadwai *et al*.[17] also reported 69.5% of isolates as ETH-resistant without any mutation in *inhA* gene.[17] Morlock *et al*.[14] found *ethA* mutations distributed widely across the structural gene of *ethA*, with no single nucleotide or codon predominating in 52% of clinical isolates for which ETH MICs were 50 g/ml.[14]

Brossier *et al*.[19] studied the association of many genes and found that together *inhA* gene, *ethA* gene, and *ethR* gene, which causes down regulator of *ethA*, contributed to 81% of mutations in ETH-resistant clinical isolates.[19] Association of *ndh* or *msh* with ETH resistance was studied but found inconclusive.[7]

Although whole-genome sequencing to determine the molecular basis of ETH resistance in absence of *inhA* mutation could not be performed. At 80.5% INHA-ETH correlation, the study provides data unique to the North Indian region, which can guide clinicians for designing individualized treatment regimens among patients in the region. Our study also emphasizes the importance of performing more studies on isolates from this region to identify all possible hot-spot regions conferring ETH resistance in *ethA*, *ethR*, and structural region of *inhA* as done from other regions.[3,19] Information obtained will guide designing molecular tests by incorporating crucial mutations for accurate detection of ETH resistance.

In 21/130 (16.1%) of *inhA* gene mutation, strains were found to be phenotypically susceptible to ETH. Phenotypic DST for ETH has a challenge that ETH drug is prone to thermolability with the possibility of degradation on incubation giving sensitive results.[21] For such strains, *inhA* resistance alone should be considered as indicative of ETH resistance

CONCLUSIONS

The correlation between *inhA* mutation and ETH resistance in the present study was 80% and based on the evidence above, the utility of *inhA* mutation in LPA for predicting ETH resistance is emphasized. Any mutation in *inhA* will guide clinicians to exclude ETH in anti-TB treatment regimens, even if phenotypic susceptible to ETH as seen in 16% cases above. In case of no mutation in *inhA* in LPA, it would be essential to perform phenotypic susceptibility for ETH at proper concentration for any resistance along with whole-genome analysis for significant mutations.

Future research needs to focus on the improvisation of molecular technologies by incorporating more genes for better detection of ETH resistance. More evidence needs to be generated by taking a larger sample size and performing multicentric studies involving both genotypic and phenotypic technologies for the detection of ETH resistance to make policy decisions at the national level in the Indian context.

Acknowledgment

The authors would like to thank technicians of the department of microbiology for performing the experiments.

Financial support and sponsorship
Nil.

Conflicts of interest
There are no conflicts of interest.

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