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Prevalence of mutations in genes associated with isoniazid resistance *Mycobacterium tuberculosis* isolates from retreated smear positive pulmonary tuberculosis patients: A Meta-analysis

Running title: Prevalence of MDR Tuberculosis

Journal category: Basic Clinical Microbiology

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Highlights

- Total 71.0% mutations were observed in katG region whereas which is more prevalent in comparison with *inhA* gene 29.0%
- The most frequent mutations (63%) were observed in MUT1probe of katG gene
- Total 19.9% mutations were observed in inhA promotor region

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- The meta-analysis derived a pooled katGS315T resistant TB prevalence of 64.5% (95% CI; 0.593 ± 0.754%) with Q value 732.19, I^2 98.35% and p-0.000 for treated TB cases.
- Mutations at T8C (6, 0.3%) and T8A (8, 0.4%) have not been published in previous studies.

Abstract: Objective: The prevalence of isoniazid mono resistance is high in India. We investigated the molecular epidemiological characteristics association with the isoniazid resistance mutations in Mycobacterium tuberculosis in codon katG315 and in the promoter region of the inhA gene.

Methods: Sputum specimens of smear-positive tuberculosis patients were subjected to Genotype MTBDRplus testing to identify katG and inhA mutations. Seventeen publications along with this current study assessed 14,100 genotypically resistant isolates for mutations in katG inclusive of codon position 315.

Results: In total, 1821 of 15438 isoniazid-resistant strains (11.8%) had detectable mutations: 71.0% in katG codon 315 (katG315) and 29.0% in the inhA promoter region. Economically active age group had 89.1%, paediatric age group had 0.4% and in the age group >60 years had 10.5% isoniazid mono resistant and in males and females were 17.7% and 15.9% respectively. The meta-analysis derived a pooled katGS315T resistant TB prevalence of 64.5% (95% CI; 0.593 ± 0.754%) with Q value 732.19, I2 98.35% and p-0.000 for treated TB cases.

Conclusion: Isoniazid resistant was transferred widely and its prevalence and transmission of INH resistant isolates especially with katG315Thr mutation was confirmed. Therefore, it is important to diagnose the katG315Thr mutants among INH-resistant strains as it could be seen as a risk factor for subsequent development of MDR-TB. Prompt detection of the patients with INH resistant strains would expedite the modification of treatment regimens

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and appropriate infection control measures could be taken in time to diminish the risk of further development and transmission of MDR-TB.

Keywords: Isoniazid resistant: Mycobacterium tuberculosis: Mutation: Promotor region

1.0 Introduction

Tuberculosis (TB) still remains an important infectious disease and public health concern worldwide. Drug resistance emerges as a result of spontaneous gene mutations in *M.tuberculosis* that renders the bacteria resistant to the most commonly used anti-TB drugs[1] thus jeopardizing the tuberculosis control activity.The prevalence of mono-resistance to isoniazid, one of the most potent first-line anti-TB agents, has been reported in ranges from 4-12% for all TBcases with a global average of 8.1% for new TB cases [2, 3].

Isoniazid (INH) is abactericidal agent that plays an essential role in short-course treatment regimens. INH is a prodrug that must be metabolized by mycobacterial catalase-peroxidase to exert its antibacterial activity. Most INH resistance in clinical isolates- resulting in blocking prodrug activation through mutations in the gene katG that alter or eliminate mycobacterial catalase-peroxidase activity [4]. Although katG insertions, deletions, and frame shifts do occur occasionally and induce complete loss of the functional gene product and correspondingly high levels of INH resistance, the majority of the mutations identified in clinical isolates are single point mutations that have resulted in intermediate levels of resistance. The INH resistance mutation that has most commonly found in clinical isolates is Ser315Thr point mutation in katG. The mutant gene product recognizes a reduced capacity for prodrug activation while retaining much of the catalase-peroxidase activity of the wild-type enzyme [5, 6].

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No such systematic review has assessed the geographic variability of the most widespread mutations associated with INH resistance but it is critically imperative to understand the frequency and geographic distribution of mutations associated with INH resistance. A failure to account for these variations limits the local effectiveness of molecular diagnostic tools currently available and constrains the development to approved genotypic diagnostic tests [7]. The aims of this systematic review are to quantify the frequency and co-occurrence of the most common mutations associated with genotypic INH drug resistance and to describe the regional differences in the distribution of these mutations.

2.0 Materials and Methods

2.1 Specimen collection and processing

The study was conducted in the Intermediate Reference Laboratory at Government Hospital for Chest Diseases, Puducherry for a total period of 42 months between July 2012 and December 2015.The sputum samples of MDR-TB suspect patientsfrom the nine districts of Tamil Nadu along with Puducherry state were collected in 50 ml clean sterile falcon tubes and transported to Intermediate Reference Laboratory at Government Hospital for Chest Diseases,Puducherrythrough cold chain mechanism as per the criteria of Revised National Tuberculosis Control Programme, India. Total 15438 sputum samples were collected from various age groups for this study, which included male 13117 and female 2321. Each sputum sample received at Intermediate Reference Laboratory was assigned lab number and consecutively screened for acid fast bacilli (AFBs) using Fluorescence (FM) smear microscopy [8].The smear positive sputum samples were directly processed by GenoType MTBDRplus assay V.2.0 (Hain Life Sciences) andall the smear negative samples were processed in BACTEC MGIT 960 system. The culture positive tubes of smear negative samples were also processed by GenoType MTBDRplus assay V.2.0. All laboratory bench works relating to potentially infectious clinical specimens were performed in a Class II

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biosafety cabinet at Bio Safety Level III facility. All remaining processed specimens were stored at -20°C for the duration of the study to allow for re-testing of specimens giving discrepant results.

2.2 GenoType MTBDRplus assay V.2.0

The GenoType MTBDRplus V.2.0 assay was performed according to the manufacturer's protocol (Hain Life science GmbH)[9]. The test is based on DNA strip technology and has three steps: DNA extraction, multiplex PCR amplification, and reverse hybridization. All three steps were performed as per the WHO recommendations [10].

2.3 Primary Culture Inoculation and Identification of Mycobacterial Species

All sputum specimens were digested and decontaminated by the standard *N*-acetyl-L-cysteine-NaOH method [11].The sediment was suspended in 1ml of sterile phosphate buffered saline (pH 6.8) and 0.5ml of the processed specimen was then inoculated into MGIT 960 vials supplemented as prescribed by the manufacturer [12].All inoculated MGIT tubes were incubated in the MGIT 960 instrument either until they were flagged positive by the instrument or for a maximum of six weeks. All positive MGIT vials were confirmed for acid fast bacilli by FM staining and further subjected to identification of *M.tuberculosis* complex by rapid immunochromatographic test for the detection of Antigen MPT64 Mycobacterium tuberculosis complex in liquid cultures. Some portion of the processed specimens was refrigerated for further use to rule out any discrepant results. Species identification was done based on observation of cultural characteristics and biochemical tests. Procedures were adopted to differentiate mycobacteria at species level only [13].

2.4 Statistical analysis

Seventeen publications including this current study assessed 14,100 genotypically resistant isolates for mutations in katG inclusive of codon position 315 [14-30].Pooled prevalence and 95% confidence intervals (95% CIs) were calculated using random effects

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model based on the exact binomial method of Hamza et al fitted Stata 13 (Stata Corp, College Station, TX)[31]. The H^2 based Q statistic and I^2 tests were used to assess the between-study heterogeneity using two-sided P-values. Begg rank correlation test and forest plot were used to assess publication bias using the Stata 13 (Stata Corp, College Station, TX). Forest plots were visually assessed for heterogeneity among the studies within each index test. Using summary plots, we examined the variability in estimates and the width of the prediction region, a wider prediction region suggests more heterogeneity.

3.0 Results

Two sputum samples collected from each MDR TB suspect's sputum samples were processed for the Auromine O phenol staining. Overall, 15438 pooled Multidrug resistant suspect's sputum positive specimens were tested for Genotype MTBDR plus assay V.2.0 to perceive the pattern of drug resistance amongst the MDR suspects. Conventional BACTEC MGIT 960 procedures were performed on all smear negative MDR suspect samples, no results/invalid obtained from Genotype MTBDR plus assay V.2.0.

The GenoType MTBDRplus strip contains 17 probes, including amplification and hybridization controls to verify the test procedures. For the detection of INH resistance, one probe covers the wild-type S315 region of katG, while two others (probes katG MUTT1 and MUTT2) are designed to assess the AGC-to-ACC (S315T) and the AGC-to-ACA (S315T) mutations. Furthermore, the promoter region of the *inhA* gene is included on the new strip and encompasses the regions from positions –15 to –16 for the inhA WT1 probe and positions –8 for the *inhA* WT2 probe. Four mutations (–15C/T, –16A/G, –8T/C and –8T/A) can be targeted with the *inhA* MUT1, MUT2, MUT3A and MUT3B probes. Either absence of one or more wild-type probe(s) or the presence/staining of mutant probes are indicative of the resistant strain (Figure 1).

Total1821 isoniazid mono resistant drug resistant strains were identified by Genotype

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MTBDRplus assay V.2.0, amongst the 1821isoniazid mono resistant cases, higher frequency of mutations was observed in *katG* gene 71.0% (1292/1821) that shows it is more prevalent in comparison with *inhA* gene 29.0% (529/1821). In INH monoresistant most frequent mutations were found in *katG* WT and MUT1:S315T1 (136 cases), occurrence of mutation in MUT1 (1150 cases) probe and MUT2 probe (6 cases), in *inhA* WT1:15/-16 (138 cases), WT2:-8 (14 cases) and C15T (363 cases) regions (Table1). Surprisingly, only two strains had mutations in both the *katG* and *inhA*genes. In rare case six INH monoresistant had a MUT3A (T8C) mutation, eight had mutation at MUT3B (T8A) and these have not been published in previous studies. Second most commonly occurring mutation in INH resistant isolates, at position-15 of the *inhA* promoter, was identified in 363 of 1821 (19.9%) phenotypically resistant and mutation in *inhA* promoter region, -8, was identified among 4.9% of phenotypically resistant isolates.

Among the MDR suspect's criteria, the isoniazid mono resistant in smear positive retreatment cases and any follow up cases were 54.6% and 28.6% respectively. The isoniazid mono resistant in known contact of MDR TB was 1% and 3.1% in HIV TB cases.The economically active age group (>15 years and < 60 years) had 89.1% of isoniazid mono resistant and paediatric age group had 0.4% isoniazid mono resistant. The isoniazid mono resistant in the age group >60 years was 10.5% as shown in Table 2.The isoniazid mono resistant in males and females were 17.7% and 15.9% (Figure 2) respectively.

The cumulative frequencies of multiple or co-occurring mutations associated with INH resistance, were first assessed by individual genes. The meta-analysis derived a pooled katGS315T resistant TB prevalence of 64.5% (95% CI; 0.593 ± 0.754%) with Q value 732.19, I^2 98.35% and p-0.000 for treated cases of TB (Figure 3). Seventeen publications have assessed 14,100 genotypically resistant isolates for mutations in katG inclusive of codon position 315. The mutation frequency for katG315 in this subset of INH resistant isolates was

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63.2%. Ninety-five percent confidence intervals (95% CI) were calculated using the random effects model based on the exact binomial method. If the 95% confidence intervals of most of the studies do not overlap line of no effect, the results are statistically significant at 5% significance levels. The 95% confidence intervals of the overall effect estimate overlap line of no effect. So, there is a statistical significance at the study level as well as at the meta-analysis level. MTBDR*plus* v2 results classified as resistant for RIF and INH were dichotomized as either resistant by MUT probe hybridization and the absence of WT probe hybridization or as resistant by the sole absence of WT probe hybridization. Resistance based on the presence of MUT probe hybridization and on the absence of WT probe hybridization was compared to resistance based both on the presence of MUT probe hybridization and the absence of WT probe hybridization and on the sole absence of WT probe hybridization by assessing the area under the receiver operating characteristic curve for equality (AUC).Frost plot analysis was performed using Stata 13 (Stata Corp, College Station, TX).

4.0 Discussion

The frequency patterns of the most common mutations associated with INH resistance appear to differ between individual genes. It is clear that the awe-inspiring majority (64%) of phenotypic INH resistance among *M.tuberculosis* isolates is associated with a single mutation, katG315.The dominance of this mutation is hypothesized to be the result of a low or zero fitness cost for this mutation, allowing it to propagate without negative selection pressure [32]. Mutations other than katG315 in the katG gene appear to occur at low (<1%) frequencies and occur overwhelmingly in conjunction with the katG315 mutation. The present study demonstrated that 71.0 % for katG315, 29.0% for inhA-15 mutations of patients with pulmonary TB had drug-resistant disease, suggesting a serious epidemic of drug-resistant tuberculosis among patients with pulmonary TB in human. This analysis showed a strong correlation between the proportion of INH resistance-conferring mutations due to

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S315T measured in clinical isolates and several different indicators of tuberculosis transmission intensity, supporting the hypothesis that mutations at the 315 position of katG confer INH resistance for *Mycobacterium tuberculosis* without diminishing virulence or transmissibility.

However, a study conducted by Lin et al., using 127 INH resistant isolates from California, a population that is thought to be mirror global MDR-TB miscellany due to immigration, identified a global frequency of 61%for katG315, 23% for inhA-15 mutations, and 83% for the cumulative frequency of either mutation, approximating the frequencies of these mutations as quantified in the systematic review [33]. In contrast, Campbell et al.,using 212 INH resistant isolates from both WHO and CDC laboratory archives estimated the global frequency of the *katG*315mutation to be 85%, *inhA*-15 to be 17%, and their cumulative frequency 91%; however, isolates used for that study were selected to provide a sundry set of mutation patterns, and therefore may not accurately represent true global frequencies [34].Finally, a more recent study conducted by Rodwell et al., using 348 INH resistant isolates from four geographically diverse countries estimated the global frequencies of katG315 and *inhA* -15to be significantly higher at 86%and 34% respectively [35].Mutations in codon 315 of the *katG* gene (*katG*315) and in the promoter region of the *inhA* gene are by far the most common. Mutations *katG*315 occur in 50 to 95% of isoniazid-resistant strains, whereas 20 to 42% of such strains have mutations in the promoter region of the *inhA* gene, depending on the geographic region studied[35].In this study, the frequency of 71.0 % for katG 315, 29.0% for inhA-15 mutations were observed, which is equal to global scenario of resistant pattern. Patterns of co-occurring mutations in the *inhA* promoter region appear to differ markedly from co-occurring mutations in the katG gene. Although the inhA-15 mutation isthedominant (19%) mutation inthe*inhA* promoter region, other resistance

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associated mutations (~1%) in the *inhA* promoter region appear to occur independently of the inhA-15mutation and frequently contribute to the detection of INH resistance.

In total, 1821 of 15438 isoniazid-resistant strains (11.8%) had detectable mutations: 71.0% in *katG* codon 315 (*katG*315) and 29.0% in the *inhA* promoter region. Resistance to isoniazid (INH) is the most common form of mono resistance with a prevalence of 10% among new tuberculosis (TB) cases and 28% among retreatment cases reported in 2009 globally [36]. INH resistance is independently associated with unfavourable treatment outcomes among smear positive retreatment TB patients, demonstrating that the current treatment regimen may be inadequate. These findings call for an exigent need for randomized controlled trials to discover the most effective treatment regimen for managing INH resistant TB. HR-TB is associated with an increased risk of treatment failure in patients who receive first-line regimens.There were no significant associations between the probability of having a strain with a *katG*315 mutation and the patient's locality, type of residence, age or presence of mutations in the *inhA* promoter region. However,*katG*315 mutations were significantly more frequent among men, among patients previously treated for tuberculosis.In conclusion, isoniazid resistance was most frequently due to mutations in the *katG*315 gene, and these mutations were also associated with multidrug and polydrug resistance, whereas *inhA* mutations were less frequent. Both *katG*315 and *inhA* mutations increased the risk of relapse.However; the cumulative frequencies of these mutations do appear to differ by region by region that could lead to variation in the sensitivity of molecular diagnostics if they are based only on these mutations. This would allow for tailoring of molecular tests to specific regions, better interpretation of the molecular tests being used, and improved therapy recommendations.

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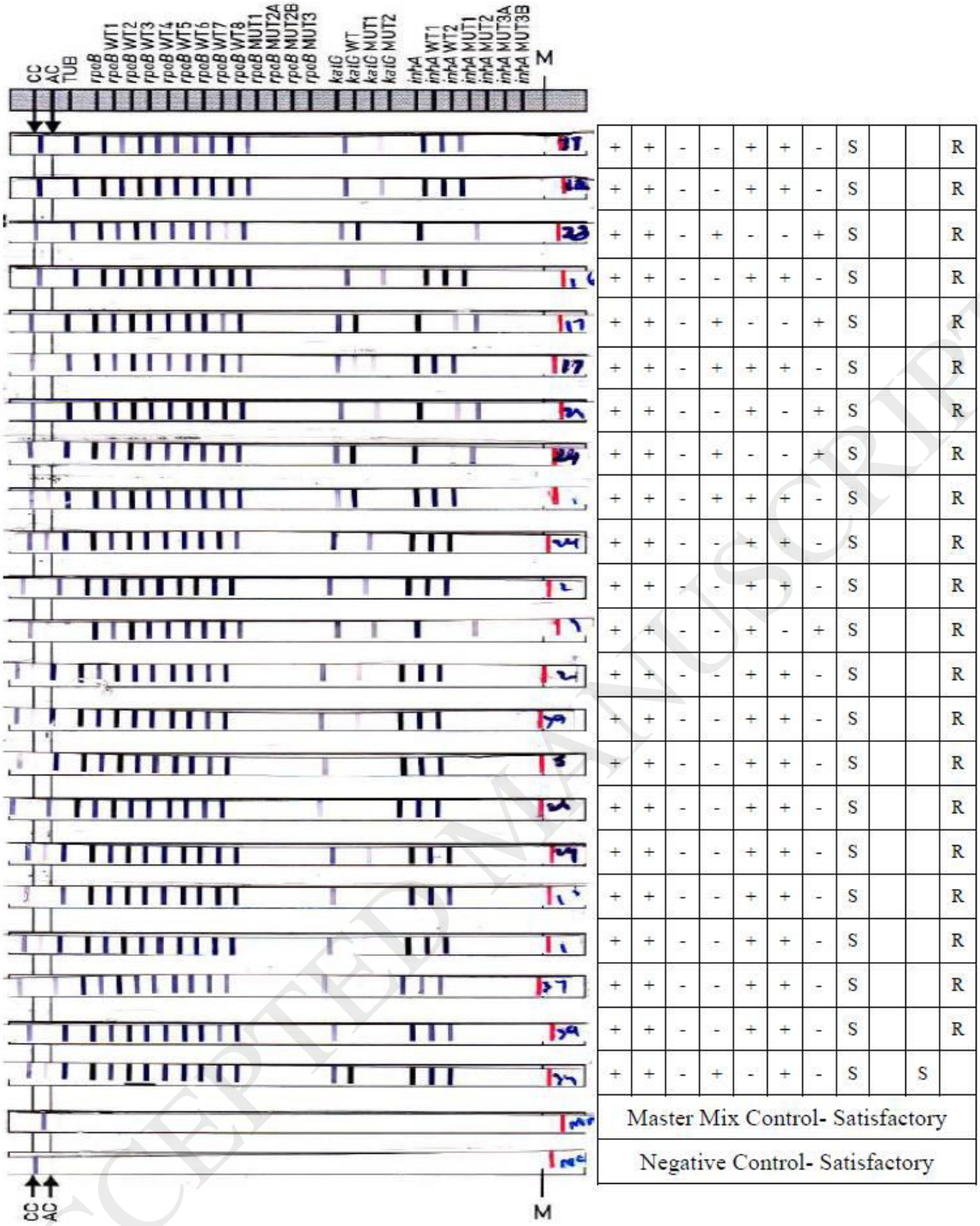

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Master Mix Control- Satisfactory										
Negative Control- Satisfactory										

Figure 1: Results of LPA with resistant pattern in *katG* and *inhA* gene

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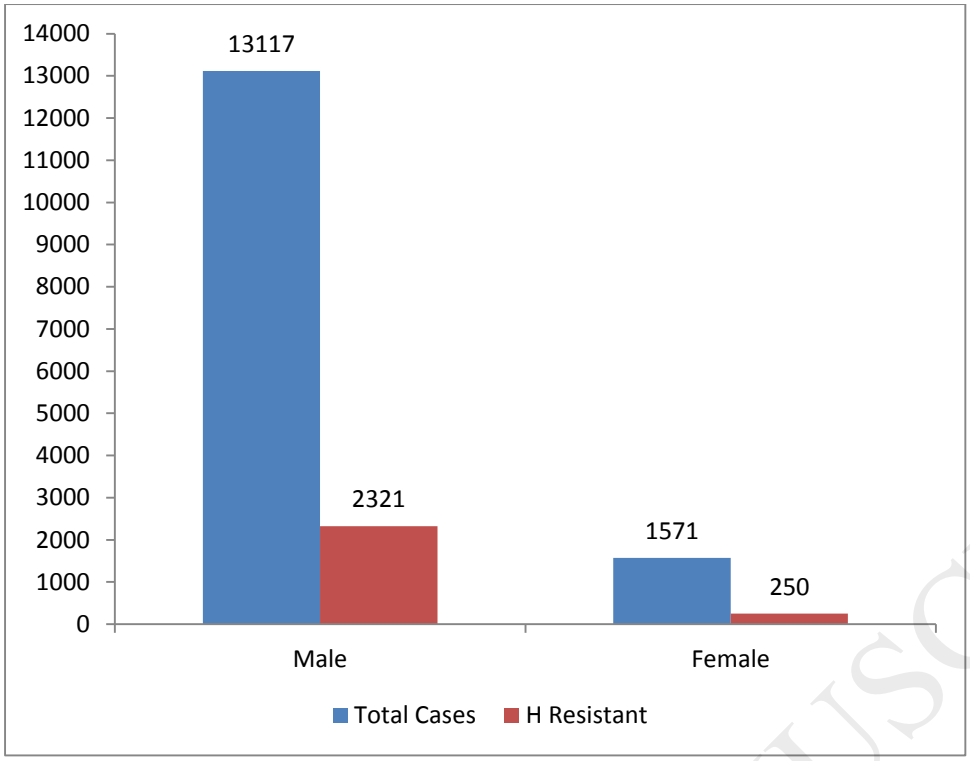

Figure 2: Results of Isoniazid mono resistant in gender wise

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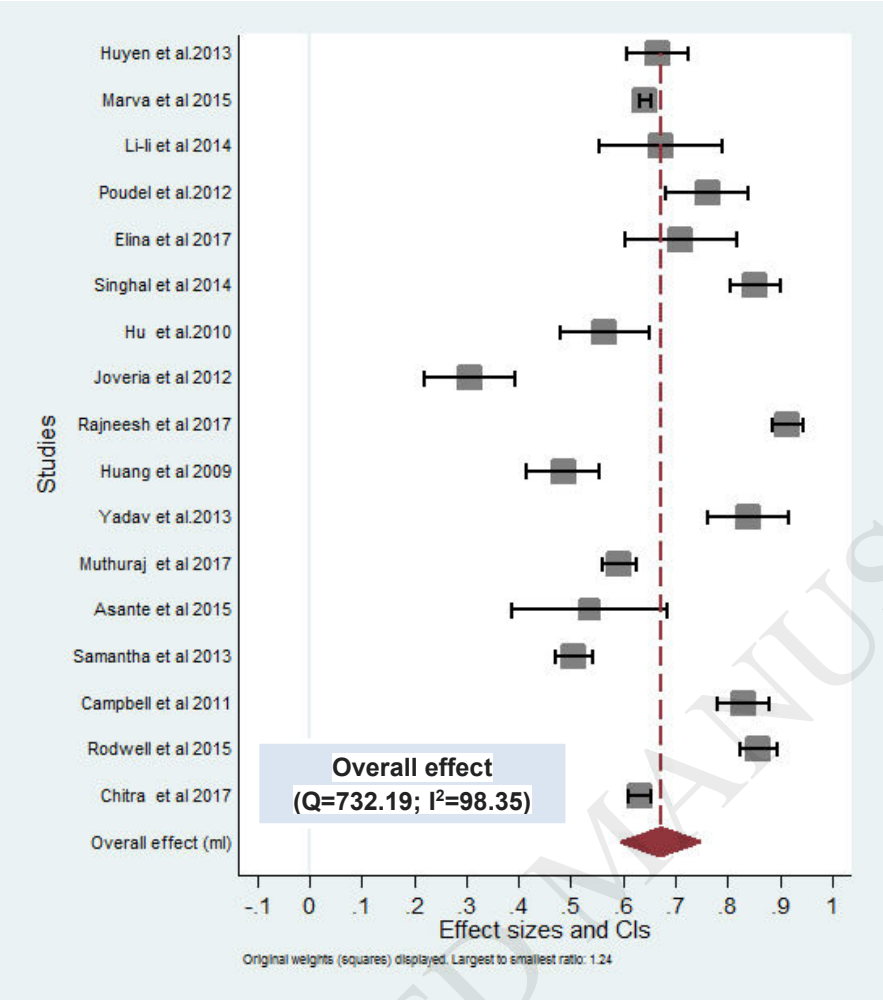
Figure 3. Forest plot of the pooled prevalence of katG S315T resistant included studies ES: effect size, 95% CI: confidence interval.



among isoniazid resistant TB cases stratified by study design of

Study	Effect	[95% Conf.	Interval]	% Weight
Huyen et al.2013	0.665	0.607	0.724	6.38
Marva et al 2015	0.642	0.631	0.652	6.59
Li-li et al 2014	0.672	0.554	0.790	5.78
Poudel et al.2012	0.761	0.682	0.840	6.21
Elina et al 2017	0.710	0.603	0.817	5.91
Singhal et al 2014	0.852	0.804	0.900	6.45
Hu et al.2010	0.565	0.480	0.650	6.15
Joveria et al 2012	0.306	0.219	0.392	6.13
Rajneesh et al 2017	0.914	0.885	0.943	6.55
Huang et al 2009	0.485	0.415	0.554	6.29
Yadav et al.2013	0.839	0.762	0.916	6.22
Muthuraj et al 2017	0.593	0.561	0.624	6.53

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Asante et al 2015	0.535	0.386	0.684	5.37
Samantha et al 2013	0.505	0.470	0.541	6.52
Campbell et al 2011	0.830	0.780	0.881	6.43
Rodwell et al 2015	0.859	0.823	0.896	6.51
Chitra et al 2017	0.632	0.609	0.654	6.18
Overall effect	0.673	0.593	0.754	100.00

Heterogeneity Measures:

	Value	df	p-value
Cochrane Q	732.19	15	0.000
I^2 (%)	98.35		
H^2	59.66		
tau^2 est	0.025		

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Table 1: Results of isoniazid resistance mutations in MDR suspect's samples on sex wise

MDR Suspects Criteria	Sex	Total case	INH Mono Resistant	katG WT	katG MUT1	katG MUT2	inhA WT1	inhA WT2	inhA MUT1	inhA MUT2	inhA MUT3A	inhA MUT3B
Failure	Male	553	71	6	46	1	4	3	10	0	1	0
	Female	94	9	1	7	0	0	0	1	0	0	0
R Rx S(+)ve at 4th month	Male	519	68	8	42	1	5	1	11	0	0	0
	Female	70	6	0	5	0	0	0	0	0	0	1
MDR TB Contact	Male	64	9	0	6	0	1	0	2	0	0	0
	Female	30	8	0	6	0	1	0	1	0	0	0
S(+) at diagnosis ,Re Rx case	Male	7694	859	65	526	3	48	10	207	0	0	0
	Female	1158	131	16	70	0	17	0	27	0	1	0
Any follow up S(+)ve	Male	3252	450	28	311	0	44	0	58	0	4	5
	Female	651	67	4	39	0	9	0	15	0	0	0
S(-)Ret Rx case	Male	579	77	7	44	0	4	0	21	0	0	1
	Female	114	10	0	8	0	2	0	0	0	0	0
HIV TB cases	Male	456	37	1	23	1	2	0	9	0	0	1
	Female	204	19	0	17	0	1	0	1	0	0	0
		15438	1821	136	1150	6	138	14	363	0	6	8

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Table 2: Results of isoniazid resistance mutations in MDR suspect's sampleson age wise

MDR TB Suspects	Sex	≤ 15 years	> 15 ≤ to 45 years	> 45 to ≤ 60 years	> 60 years	Total
Failure	Male	1	34	28	8	71
	Female	0	9	0	0	9
R Rx S(+)ve at 4th month	Male	0	41	20	7	68
	Female	0	4	2	0	6
MDR TB Contact	Male	1	6	1	1	9
	Female	2	4	1	1	8
S(+) at diagnosis,Re Rx case	Male	1	436	343	79	859
	Female	0	91	34	6	131
Any follow up S(+)ve	Male	0	220	159	71	450
	Female	0	51	8	8	67
S(-)Retreatment case	Male	1	37	30	9	77
	Female	0	6	3	1	10
HIV TB cases	Male	2	28	7	0	37
	Female	0	17	2	0	19
		8	984	638	191	1821

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Table 3: Results of Isoniazid mono resistant among the MDR suspect's patient on criteria basis

Criteria	MDR TB Suspects	INH Mono resistant	% Total ; 95% CI
Failure	647	80	12.36%; 9.9 – 15.2
R Rx S(+)ve at 4th month	589	74	12.56%; 10.0 – 15.5
MDR TB Contact	94	17	18.09%; 10.9 – 27.4
S(+) at diagnosis,Re Rx case	8852	990	11.18%; 10.5 – 11.9
Any follow up S(+)ve	3903	517	13.25%; 12.2 – 14.3
S(-)Ret Rx case	693	87	12.55%; 10.2 – 15.3
HIV TB cases	660	56	08.48%; 6.5 – 10.9

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Table 4: Results of mutations pattern of 1821 Isoniazid mono resistant TB patients

Mutation(s)		Frequency	
katG	inhA	No of patients	% Total ;95% CI
WT(315) Absent	-	136	7.5%;6.3-8.8
MUT1(S315T1)	-	1150	63.2%;60.9-65.4
MUT1(S315T2)	-	6	0.3%;0.1-0.7
-	WT(-15/-16)Absent	138	7.6%;6.4-8.9
-	WT(-8)Absent	14	0.8%;0.4-1.3
-	MUT1(C15T)	363	19.9%;18.1-21.8
-	MUT2(A16G)	0	0%; 0
-	MUT3A(T8C)	6	0.3%;0.1-0.7
-	MUT3B(T8A)	8	0.4%;0.2-0.9

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