GMR
The Original

FREQUENCY AND DISTRIBUTION OF DRUG RESISTANCE-ASSOCIATED MUTATIONS (rpoB, katG, inhA, gyrA, rrs and eis) IN *MYCOBACTERIUM TUBERCULOSIS* ISOLATES FROM SOUTHERN PUNJAB

[1]Muhammad Shahbaz Hussain, [2*]Sidrah Saleem, [3]Hafiz Muhammad Rizwan, [4]Muhammad Imran Shoukat, [5]Shah Jahan, [6]Syeda Fatima Nadeem

[1]PhD Scholar, University of Health Sciences, Lahore, Punjab, Pakistan
Email: drmshahbazhussain@gmail.com
[2]M.B.B.S, MPhil (Microbiology), PhD (Microbiology), Professor (Microbiology), University of Health Sciences Lahore, Pakistan,
Email: Sidu_78@yahoo.com
[3]Assistant Professor, Department of Pulmonology, Sheikh Zayed Medical College Hospital Rahim yar khan, Email: hafiz_rizwan_u@yahoo.com
[4]Senior Demonstrator, Pathology Department, Sheikh Zayed medical College, Rahim Yar Khan, Punjab, Pakistan,
Email: drimranshoukat83@gmail.com
[5]B.Sc., PhD (Virology), Associate Professor (AHS), University of Health Sciences Lahore, Pakistan, Email: captainmalik@hotmail.com
[6]MPhil (Microbiology), Medical Laboratory Technologist, BSL III COVID 19 Laboratory, Sheikh Zayed Medical College, Rahim Yar Khan, Pakistan , Email: fatimanadeem291@gmail.com

ABSTRACT

Background: The emergence of drug-resistant tuberculosis (DR-TB) has become a major challenge to global tuberculosis control programs. Resistance in *Mycobacterium tuberculosis* is primarily driven by mutations in specific genes associated with first-line and second-line anti-tuberculous drugs. This study aimed to determine the frequency and distribution of drug resistance-associated mutations in *M. tuberculosis* isolates from tertiary care hospitals of Southern Punjab, Pakistan.

Methods: A cross-sectional study was conducted on sputum samples collected from suspected tuberculosis patients presenting to four tertiary care hospitals in Southern Punjab, Pakistan, including Nishtar Hospital Multan, Bahawal Victoria Hospital Bahawalpur, Sheikh Zayed Hospital Rahim Yar Khan, and DHQ Hospital Dera Ghazi Khan. Molecular diagnostic techniques were employed to detect mutations associated with resistance to first-line and second-line anti-tuberculosis drugs. The analyzed mutations included **rpoB**, **katG**, **inhA**, **gyrA**, **rrs**, and **eis** genes. Statistical analysis was performed to compare mutation frequencies among study sites. **Results**: Among the detected resistance-associated mutations, **rpoB** was the most prevalent mutation, identified in 35.8% of isolates, followed by **inhA** (25.9%), **katG** (14.8%), **gyrA** (9.9%), **rrs** (8.6%), and **eis** (4.9%). Hospital-wise analysis showed comparable mutation frequencies across all study sites. The prevalence of **rpoB** mutation ranged from 34.6% to 37.5%, while **inhA** mutation ranged from 18.8% to 26.9%. No statistically significant differences were observed in the distribution of any mutation among hospitals ($p > 0.05$).

Conclusion: The predominance of **rpoB** and **inhA** mutations indicates a high burden of rifampicin and isoniazid resistance among *M. tuberculosis* isolates in Southern Punjab. The uniform distribution of resistance-associated mutations across hospitals suggests widespread circulation of resistant strains in the region. Rapid molecular detection of these mutations can facilitate early diagnosis and guide effective treatment strategies for drug-resistant tuberculosis.

KEYWORDS: Drug-resistant tuberculosis; *Mycobacterium tuberculosis*; Gene mutations; rpoB; inhA; Molecular diagnostics; Pakistan

INTRODUCTION

The disease Tuberculosis (TB) caused by Mycobacterium tuberculosis is amongst the highest killers and morbidities causing infection in the world. Although TB is a preventable and curable disease, it still poses a significant public health burden, especially in LMICs. The World Health Organization estimates that there were about 10.8 million tuberculosis cases, and more than 1 million tuberculosis deaths, worldwide each year. Again, Pakistan continues to be a high TB burden country and has a substantial contribution in the burden of drug resistant TB (DR-TB). (WHO, 2024) or (Singh et al., 2025).

New strains of TB that can no longer be treated with certain drugs – known as extensively drug-resistant TB (XDR-TB) and multidrug-resistant tuberculosis (MDR-TB) – have been a big challenge in TB control programs. MDR-TB is TB that is resistant to at least rifampicin and isoniazid, the best first-line medications against TB. XDR-TB is an even more resistant type of resistance that has two levels: firstly, resistance to fluoroquinolones, plus resistance to

second-line drugs (also known as injectable drugs). Long course of treatment, high treatment cost, low effectiveness and high mortality are related to these resistant forms (Gul et al., 2026; Baig et al., 2024).

Intrinsically ways in which drug resistance occurs in M. tuberculosis include spontaneous chromosomal mutation of genes that encode drug target proteins or enzymes that activate the drugs. HGT has not been documented as a significant pathway of resistance acquisition in M. tuberculosis as compared to many other bacteria. In this context, a molecular marker is the ability to detect certain mutations and thus, lead to the quick identification of the drug-resistant strain. (Ranjan et al., 2023; Yan et al., 2023).

Mutations in the rpoB gene have a strong correlation with rifampicin resistance and are seen as significant surrogate markers for the identification of MDR-TB. In the same way, mutations in katG and inhA genes are generally associated with isoniazid resistance. A high level isoniazid resistance is associated with mutation in katG, specifically at codon 315, and mutation in inhA generally will give low-level resistance. Mutations in gyrA gene are the major causes of resistance to fluoroquinolones and mutations in rrs genes and eis genes are common causes of resistance to second line injectable drugs (Khan et al., 2023; Soko et al., 2026).

The molecular diagnostic techniques that detect these mutations associated to resistance have transformed the way DR-TB is diagnosed. These rapid methods enable earlier detection of resistant strains than culture-based drug susceptibility testing, allowing for prompt implementation of a suitable treatment program and minimize the spread of drug resistant isolates (Aftab et al., 2023; Soidah et al., 2025).

There are distinct geographic variation in prevalence of resistance-associated mutations, depending on the resistance level, the level of strain diversity, use of antibiotics, and the strength of selection pressure. Several studies have been done worldwide of mutation frequency of drug resistant M. tuberculosis, however the frequency data in Pakistan especially Southern Punjab is limited. Local mutation profiling is crucial for local resistance patterns and for designing diagnostic protocols (Agonafir et al., 2023; Reta et al., 2023).

Hence, the present study was planned to find the prevalence and distribution of these mutations associated with drug resistance in rpoB, katG, inhA, gyrA, rrs and eis genes of Mtb collected from tertiary care hospitals of Southern Punjab, Pakistan. The results of this study could help to improve the molecular surveillance and the management of drug resistant TB in the region.

MATERIALS AND METHODS

Study Design and Setting

A multicenter cross-sectional study was conducted to investigate the frequency and distribution of drug resistance-associated mutations in *Mycobacterium tuberculosis* isolates from Southern Punjab, Pakistan. The study was carried out at four major tertiary care hospitals, including **Nishtar Hospital Multan**, **Bahawal Victoria Hospital Bahawalpur**, **Sheikh Zayed Hospital Rahim Yar Khan**, and **DHQ Teaching Hospital Dera Ghazi Khan**. These centers serve as major referral facilities for tuberculosis diagnosis and management across Southern Punjab.

Study Population and Sample Collection

Sputum samples were collected from clinically suspected pulmonary tuberculosis patients presenting to the participating hospitals during the study period. Patients with symptoms suggestive of pulmonary tuberculosis, including persistent cough, fever, weight loss, hemoptysis, and night sweats, were included in the study. Duplicate samples, contaminated specimens, and inadequate sputum samples were excluded.

Samples were collected in sterile leak-proof containers following standard biosafety guidelines and transported to the laboratory under controlled conditions for further processing.

Sample Processing and Identification

All sputum specimens were processed using standard mycobacteriological procedures. Decontamination and liquefaction were performed using the **NALC–NaOH method** to eliminate contaminating flora while preserving viable mycobacteria. Processed samples were centrifuged, and pellets were used for subsequent molecular analysis.

Initial detection of *Mycobacterium tuberculosis* complex was performed using rapid molecular diagnostic assays.

Samples negative for *M. tuberculosis* but showing mycobacterial growth were further evaluated for possible non-tuberculous mycobacteria (NTM).

Molecular Detection of Drug Resistance-Associated Mutations

Genotypic analysis of resistance-associated mutations was performed using molecular diagnostic techniques targeting genes associated with resistance to first-line and second-line anti-tuberculous drugs.

The following genes were analyzed:

- **rpoB** → rifampicin resistance
- **katG** and **inhA** → isoniazid resistance
- **gyrA** → fluoroquinolone resistance
- **rrs** and **eis** → second-line injectable drug resistance

DNA was extracted from processed specimens according to manufacturer-recommended protocols. Amplification and hybridization were performed using standardized procedures to identify mutation-specific and wild-type probe patterns. Presence of mutation bands or absence of wild-type bands was interpreted as evidence of genetic resistance.

Classification of Drug Resistance

Drug resistance patterns were categorized according to internationally accepted definitions.

- **Drug-sensitive TB:** susceptible to first-line drugs
- **MDR-TB:** resistant to at least rifampicin and isoniazid
- **Pre-XDR-TB:** MDR-TB with additional resistance to any fluoroquinolone
- **XDR-TB:** MDR-TB with additional resistance to fluoroquinolones and at least one second-line injectable drug

Mutation profiles were recorded for each isolate and correlated with resistance patterns.

Data Collection and Statistical Analysis

Clinical, demographic, and molecular data were entered into a structured database and analyzed using **IBM SPSS Statistics version 26.0**. Categorical variables were presented as frequencies and percentages.

Comparisons of mutation frequencies among the four participating hospitals were performed using the **Chi-square test** or **Fisher's exact test**, where appropriate. A p-value of **<0.05** was considered statistically significant.

Ethical Considerations

The study was conducted in accordance with the principles of the **Declaration of Helsinki**. Ethical approval was obtained from the relevant institutional ethical review committee prior to study initiation. Confidentiality of patient data was strictly maintained, and all specimens were anonymized before analysis.

RESULTS

Distribution of Drug Resistance-Associated Mutations

A total of *Mycobacterium tuberculosis* isolates obtained from four tertiary care hospitals of Southern Punjab were analyzed for mutations associated with resistance to first-line and second-line anti-tuberculous drugs. Mutations were detected in six target genes: **rpoB, katG, inhA, gyrA, rrs** and **eis**.

Overall, **rpoB** mutation was the most frequently detected (35.8%), followed by **inhA** (25.9%), **katG** (14.8%), **gyrA** (9.9%), **rrs** (8.6%) and **eis** (4.9%) (Figure 1) (Gul et al., 2026; Xu et al., 2025).

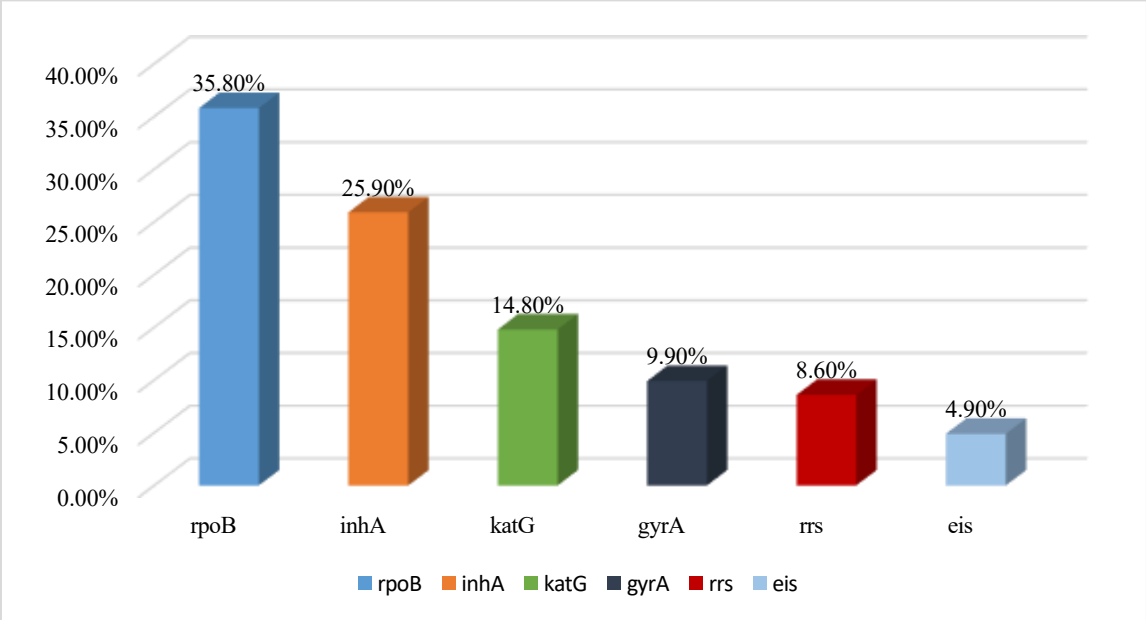
Hospital-wise comparison showed no statistically significant differences in the frequency of any mutation among the four centers (all $p > 0.05$), indicating a relatively uniform distribution of resistance-associated mutations across Southern Punjab (Table 1; Figure 2). (Singh et al., 2025).

Table 1 Distribution of drug resistance-associated mutations among Mycobacterium tuberculosis isolates from four tertiary care hospitals

Gene (Associated Drug)	NH Multan (n=81)	BVH Bahawalpur (n=36)	SZH Rahim Yar Khan (n=26)	DG Khan (n=32)	p-value*
rpoB (Rifampicin)	35.8% (29)	36.1% (13)	34.6% (9)	37.5% (12)	>0.999
katG (Isoniazid)	14.8% (12)	13.9% (5)	11.5% (3)	12.5% (4)	>0.999
inhA (Isoniazid)	25.9% (21)	25.0% (9)	26.9% (7)	18.8% (6)	0.952
gyrA (Fluoroquinolones)	9.9% (8)	11.1% (4)	11.5% (3)	12.5% (4)	0.961
rrs (Injectable drugs)	8.6% (7)	8.3% (3)	7.7% (2)	12.5% (4)	0.948
eis (Injectable drugs)	4.9% (4)	5.6% (2)	7.7% (2)	6.3% (2)	0.953

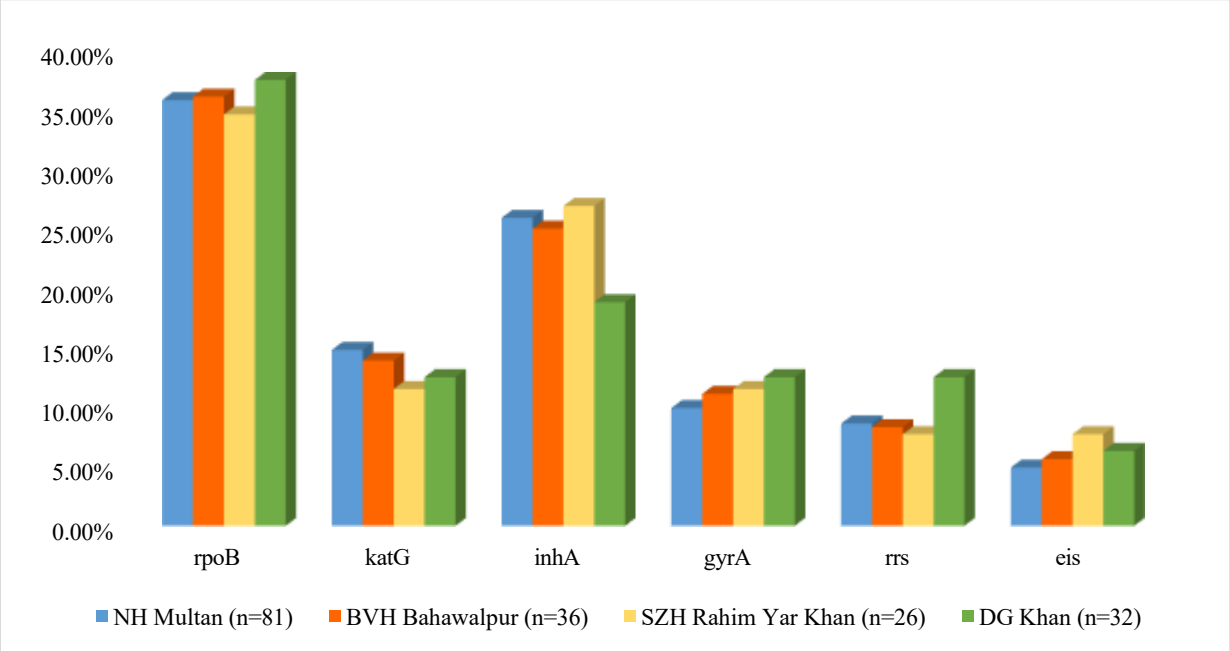
*p-value calculated using Chi-square test/Fisher's exact test as appropriate.
NH: Nishtar Hospital; **BVH:** Bahawal Victoria Hospital; **SZH:** Sheikh Zayed Hospital; **DG Khan:** DHQ Teaching Hospital Dera Ghazi Khan.

Figure 1: Bar chart showing overall frequency (%) of mutations (n=175)



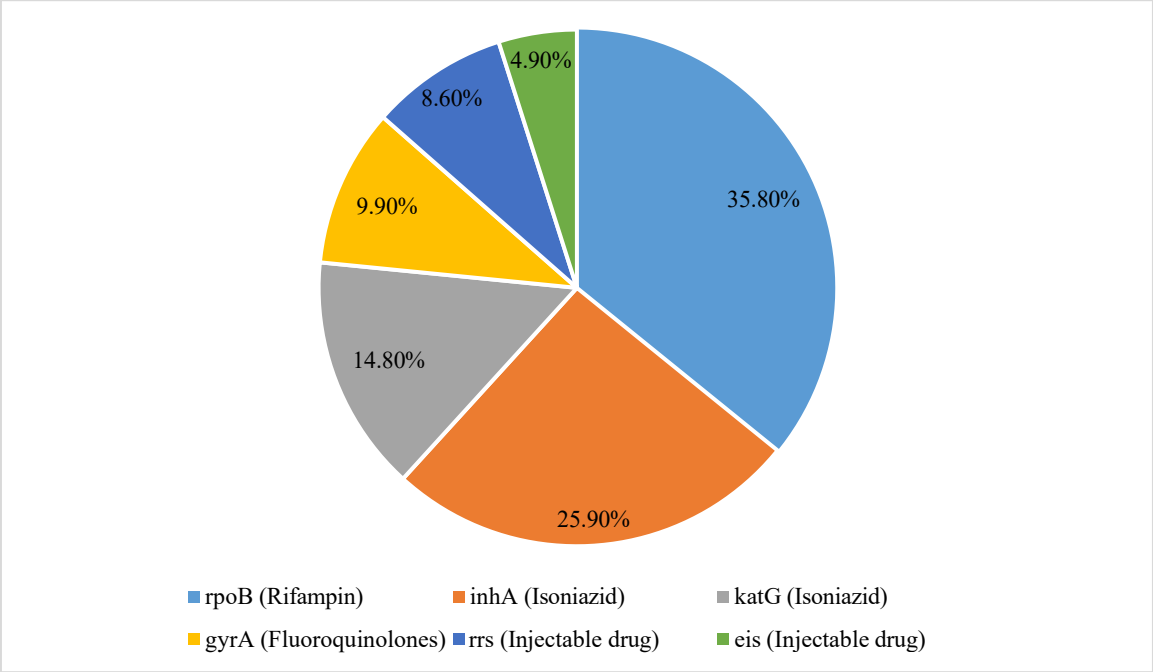
rpoB was the most prevalent mutation, followed by inhA and katG, while eis mutation was the least commonly detected.

Figure 2: Hospital-wise Mutation Frequencies (%)



No statistically significant differences were observed in the mutation frequencies among the four hospitals (all $p > 0.05$).

Figure 3: Proportional contribution of different mutations - n=175



A majority (61.7%) of mutations were associated with resistance to first-line drugs (rpoB, inhA and katG), while 38.3% were associated with second-line drugs.

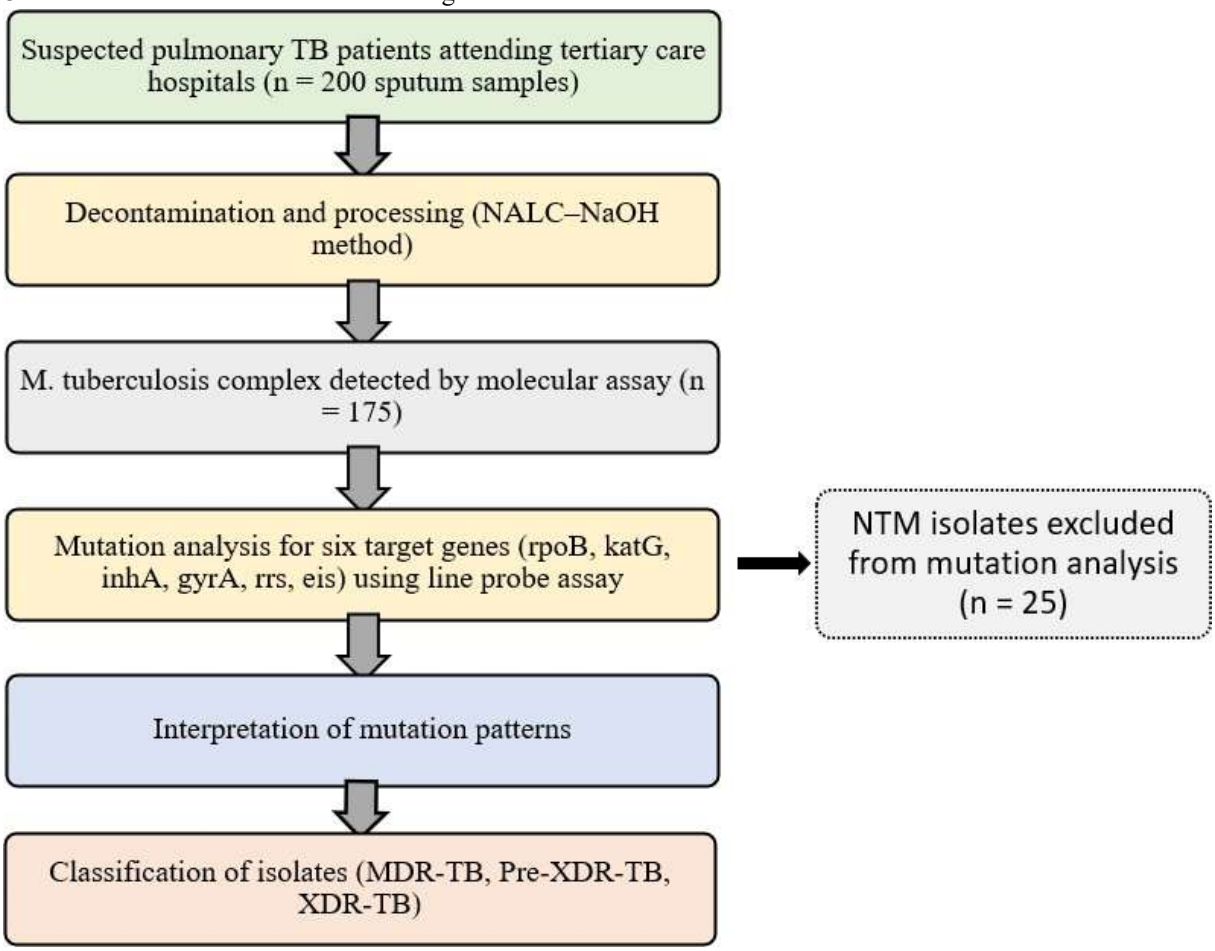


Figure 4: Flow diagram of study process

DISCUSSION

As emergence of DR-TB remains a major hurdle in the path of global TB decrease it is of critical importance in high-burden countries like Pakistan. The mutation profile of Mycobacterium tuberculosis (Mtb) from Southern Punjab in

the present study revealed that rpoB had the highest frequency of mutations associated with resistance followed by inhA and katG. The predominant resistance distribution of RIF and INH is radiated from these results.

In this study, the high frequency of mutations in the rpoB gene is similar to that previously reported at regional and international levels where this gene was found to be the major marker for rifampicin resistance. The presence of large numbers of rpoB mutations indicates that there is a significant burden of MDR-TB in Southern Punjab, as rpoB resistance is considered a strong surrogate marker of MDR-TB. Mutations in the Rifampicin resistance determining region (RRDR) of rpoB commonly occur at codons 516, 526 and 531 and are well reported as being the major factors in phenotypic resistance. (Khan et al., 2023; Soko et al., 2026).

An important discovery for the present study was that the incidence of inhA mutations was higher than katG mutations. Many studies have reported katG as the predominant mutation for isoniazid resistance, especially transition of Ser to Thr at position 315. Single nucleotide polymorphisms (SNPs) of the inhA gene, however, were significantly correlated in this study and may suggest greater circulation of low-level isoniazid resistant strains or longer duration of exposure to Isoniazid-based regimens in the local population. This is also a possibility for the influence of treatment non-compliance, interrupted therapy or inappropriate antibiotic therapy (selective drug pressure) (Ranjan et al., 2023; Yan et al., 2023)

Analogous to this, rrs and eis mutations, though not widespread, are also clinically significant because they also have been linked to resistance to second line injectable treatment. The lower rate of eis mutation obtained in the present study, is not very divergent from the literature which indicates that resistance by promoter mutation to eis is less frequent than other modes of resistance. But, these mutations still warrant concern for future expansion of extensively drug resistant strains. (Nguyen et al., 2023; Qadir et al., 2024).

One of the major limitations in this study was that it was conducted in four major tertiary care hospitals situated in Southern Punjab (multicenter data design). The mutation frequencies were found to be very consistent over the study sites with no statistically significant differences between the hospitals. It indicates that the drug resistant M. tuberculosis are not confined to transmission hotspots, but might be extensively spread through the region (Agonafir et al., 2023; Reta et al., 2023).

The uniform mutation landscape may be due to common forces bringing about the mutations such as late diagnosis, treatment failure, poor adherence and continued spread of resistant strains in the community. These results help to highlight the importance of TB surveillance on a regional scale, and not just at the individual hospital level.

Conventional drug susceptibility testing using culture can be cumbersome, whereas rapid molecular diagnostics for the detection of resistance associated mutations has many advantages. Gene mutations in rpoB, katG, inhA, gyrA, rrs, and eis can be detected early, which in turn can help to speed up delayed initiates of personalized treatment regimens to stop transmission and enhance treatment outcomes. Implementation of molecular surveillance in regular TB control programme can significantly enhance DR-TB management in Pakistan (Aftab et al., 2023; Baig et al., 2024).

This study has some limitations. Study was limited to a few genes for resistance, and may not identify rare and new mutations beyond the scope of the study's genomic regions. Furthermore, there was no whole genome sequencing in order to perform deeper phylogenetic analysis of resistant strains. Advances media sequencing approaches could offer more detailed analyses of resistance evolution and transmission dynamics in the future (Soidah et al., 2025).

CONCLUSION

In the present study, a heavy burden of drug resistance associated mutations in the isolates of Mycobacterium tuberculosis (MTB) has been detected in the southern part of Punjab, Pakistan. The gene mutations that were found in rpoB and inhA were the most common, suggesting that there is high level of resistance to both drugs in the circulating strains. Although the frequencies of the second-line drug resistance mutations gyrA, rrs and eis were low, they present a concern for the continued emergence of pre-XDR and XDR tuberculosis (Gul et al., 2026; Singh et al., 2025). The comparable pattern of mutations found in the all the participating tertiary care hospitals indicate that the spread of resistant M. tuberculosis strains is regionalized instead of localized spreading. The results highlight the importance of strengthening molecular surveillance, rapid detection of early resistant strains and prompt inculcation of targeted therapy. The timely use of rapid MDTs in TB control programmes has the potential to significantly enhance case detection, optimize TB treatment strategies, and limit spread of the resistant TB strains. Therefore, Pakistan (Liu et al., 2024; Xu et al., 2025) needs more surveillance action and constant monitoring of the drug-resistant mutations.

REFERENCES

1. Agonafir, M. et al. (2023) *Profile and Frequency of Mutations Conferring Drug-Resistance in Mycobacterium tuberculosis*. University of Pretoria Repository.
2. Aftab, A. et al. (2023) 'Early detection of MDR Mycobacterium tuberculosis mutations', *Pakistan Journal of Medical Sciences*.
3. Baig, S. et al. (2024) 'MDR TB – The Lethal Sound of Silent Mutations', *Journal of the College of Physicians and Surgeons Pakistan*.
4. Gul, M. et al. (2026) 'Genetic Characterization of First-Line Drug-Resistance Mutations in Mycobacterium tuberculosis Isolates', *Pathogens*, 15(5), p. 455.

5. Khan, A.S. et al. (2023) 'Large-scale statistical analysis of Mycobacterium tuberculosis mutations', *Scientific Reports*.
6. Liu, H. et al. (2024) 'Influencing factors of rifampicin resistance and analysis of rpoB gene mutations', *Journal of Clinical Tuberculosis and Other Mycobacterial Diseases*.
7. Nguyen, T.B.N. et al. (2023) 'Drug resistance and the genotypic characteristics of rpoB gene', *Osong Public Health and Research Perspectives*.
8. Qadir, M. et al. (2024) 'Profiling and identification of novel rpoB mutations', *Journal of Infection and Chemotherapy*.
9. Ranjan, K.P. et al. (2023) 'Molecular Characterization of katG and inhA Mutations by Genotype MTBDRplus', *Cureus*.
10. Reta, M.A. et al. (2023) 'Prevalence of drug resistance mutations in TB', *International Journal of Infectious Diseases*.
11. Singh, A.K. et al. (2025) 'Molecular insights of drug-resistant tuberculosis: genetic mutations in rpoB, katG and inhA', *Frontiers in Microbiology*.
12. Soidah, S. et al. (2025) 'Beyond Hotspot Mutations: Diagnostic Relevance of High-Confidence Mutations', *Journal of Clinical Microbiology*.
13. Soko, S. et al. (2026) 'Analysis of rpoB mutations associated with rifampicin resistance', *BMC Infectious Diseases*.
14. Xu, Y. et al. (2025) 'Drug resistance profile of Mycobacterium tuberculosis', *Frontiers in Microbiology*.
15. Yan, H.Y. et al. (2023) 'Characterization of Mutations in Genes Associated with Drug Resistance', *Biomedical and Environmental Sciences*