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MOLECULAR EPIDEMIOLOGY OF DRUG RESISTANCE-ASSOCIATED MUTATIONS (RPOB, KATG, INHA, GYRA, RRS, AND EIS) IN MYCOBACTERIUM TUBERCULOSIS ISOLATES

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ABSTRACT

Background: Drug-resistant tuberculosis (DR-TB) constitutes a critical public health emergency in Pakistan, with Sindh province bearing a disproportionately high burden. However, comprehensive province-level molecular data on resistance-conferring mutations remain scarce. This study aimed to determine the frequency, spectrum, and distribution of drug resistance-associated mutations (*rpoB*, *katG*, *inhA*, *gyrA*, *rrs*, and *eis*) in *Mycobacterium tuberculosis* (MTB) isolates from Sindh, Pakistan.

Methods: A hospital-based, multicentre, cross-sectional study was conducted across four tertiary-care hospitals (JPMC and Civil Hospital/DUHS, Karachi; LUH, Hyderabad; PMUH, Nawabshah). A total of 195 MTB isolates from clinically suspected pulmonary TB patients were processed using the NALC–NaOH method and confirmed by GeneXpert MTB/RIF Ultra. Resistance-associated mutations were detected by line probe assay targeting the six resistance genes. Data were analysed using IBM SPSS version 26.0; $p < 0.05$ was considered statistically significant.

Results: The overall mutational frequencies were: *rpoB* 37.9% (74/195), *inhA* 22.1% (43/195), *katG* 16.4% (32/195), *gyrA* 11.8% (23/195), *rrs* 9.2% (18/195), and *eis* 5.6% (11/195). The predominant mutations were *rpoB* S531L, *katG* S315T, *inhA* −15 C→T, *gyrA* D94G, *rrs* A1401G, and *eis* −14 C→T. Genotypic MDR-TB was identified in 31.8% (62/195) of isolates, while pre-XDR and XDR profiles accounted for 5.6% (11/195) and 6.2% (12/195), respectively. Mutation frequencies did not differ significantly across the four hospitals ($p > 0.05$), indicating homogeneous province-wide dissemination of resistant strains. Previously treated patients exhibited a significantly higher resistance burden than new cases ($p < 0.001$).

Conclusion: The high prevalence of first- and second-line resistance mutations confirms the extensive circulation of MDR, pre-XDR, and XDR-TB strains across Sindh. These findings support the expanded deployment of second-line molecular diagnostics, continuous genomic surveillance, and strengthened programmatic interventions, and provide a province-specific baseline for diagnostic validation and evidence-based treatment policy.

KEYWORDS: Mycobacterium tuberculosis; drug resistance; molecular epidemiology; rpoB; katG; inhA; gyrA; rrs; eis; MDR-TB; XDR-TB; Sindh; Pakistan.

INTRODUCTION

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (MTB), remains one of the most devastating infectious diseases worldwide, constituting a persistent public health emergency despite decades of control efforts. The World Health Organization (WHO) Global Tuberculosis Report estimates that approximately 10.6 million individuals developed TB globally in recent reporting periods, with an estimated 1.3–1.6 million TB-related deaths annually, cementing its position as the leading cause of mortality from a single infectious agent (WHO, 2023; 2024). The emergence and global dissemination of drug-resistant TB (DR-TB), particularly multidrug-resistant TB (MDR-TB; resistance to at least isoniazid and rifampicin) and extensively drug-resistant TB (XDR-TB; MDR plus resistance to any fluoroquinolone and at least one second-line injectable agent or bedaquiline/linezolid), have profoundly complicated treatment paradigms, inflated healthcare costs, and worsened patient outcomes (WHO, 2024; Dheda et al., 2017).

Pakistan occupies a critical position in the global TB landscape, consistently ranking among the five to six highest

TB-burden countries worldwide. The country accounts for an estimated 5.8–6.2% of the global TB incidence, with an annual notification rate that substantially underestimates the true disease burden owing to diagnostic gaps, fragmented healthcare delivery, and underreporting from the private sector (WHO, 2023; Pakistan National TB Control Programme, 2023). The estimated prevalence of MDR/RR-TB in Pakistan ranges between 4–5% among new cases and 15–20% among previously treated cases, figures that signal an escalating resistance crisis (Afaq et al., 2020; Qadeer et al., 2021). Furthermore, pre-XDR and XDR-TB strains have been increasingly identified in tertiary-care centres across major urban areas, raising urgent alarm for the national TB control programme (Hasan et al., 2019; Irfan et al., 2022).

Sindh, the second-most populous province of Pakistan, bears a disproportionately heavy share of the national TB burden. The province, home to over 55 million people, is characterised by dense urban agglomerations—most notably Karachi, the country's largest metropolis and a well-documented hotspot for drug-resistant TB—alongside vast rural hinterlands with limited diagnostic and therapeutic infrastructure (Khan et al., 2020; Sindh TB Control Programme Annual Report, 2022). Karachi alone contributes a substantial fraction of Pakistan's notified TB cases and has been identified in multiple studies as a reservoir for MDR-TB and XDR-TB transmission, fuelled by overcrowding, poverty, incomplete treatment adherence, unregulated antibiotic dispensing, and the presence of a large private healthcare sector operating outside the national DOTS (Directly Observed Treatment, Short-course) framework (Hasan et al., 2019; Rizvi et al., 2021). Sindh also experiences high rates of loss to follow-up, treatment failure, and empirical or sub-optimal regimens, all of which are potent drivers of acquired drug resistance (Amanullah et al., 2020).

At the molecular level, drug resistance in *M. tuberculosis* arises primarily through chromosomal mutations in genes encoding drug targets or drug-activating enzymes, rather than through horizontally acquired resistance determinants. A well-characterized constellation of genetic loci underpins resistance to first- and second-line anti-TB agents: Rifampicin resistance is conferred in >95% of cases by mutations within the 81-bp rifampicin resistance-determining region (RRDR) of the *rpoB* gene, which encodes the β-subunit of DNA-dependent RNA polymerase. Codons 526 (His→Tyr/Asp) and 531 (Ser→Leu) account for the majority of rifampicin-resistant isolates globally and in South Asian settings (Ramaswamy & Musser, 1998; Zignol et al., 2016). Isoniazid resistance is genetically more heterogeneous. The most prevalent mechanism involves mutations in the *katG* gene (encoding catalase-peroxidase, which activates the prodrug isoniazid), with the S315T substitution in codon 315 accounting for approximately 50–90% of isoniazid-resistant isolates depending on the geographical setting (Heym et al., 1995; Van Rie et al., 2001). Additional resistance arises from mutations in the promoter region of the *inhA* gene (encoding enoyl-ACP reductase, the drug's target), particularly at positions −15 (C→T) and −8 (C→T), which upregulate *inhA* expression and confer low-level isoniazid resistance, often with cross-resistance to ethionamide (Vilchèze et al., 2018). Fluoroquinolone resistance, a defining feature of pre-XDR and XDR-TB, is predominantly mediated by mutations in the quinolone resistance-determining region (QRDR) of the *gyrA* gene, encoding the A-subunit of DNA gyrase. Codons 90, 91, and 94 (particularly D94G, A90V, and S91P) are the most frequently implicated positions (Takiff et al., 1994; Malik et al., 2012). Resistance to second-line injectable agents (amikacin, kanamycin, and capreomycin) is associated with mutations in the *rrs* gene (encoding 16S ribosomal RNA), especially at position 1401 (A→G), and in the promoter region of the *eis* gene (encoding an aminoglycoside acetyltransferase), where mutations at positions −14, −10, −37, and −12 modulate *eis* expression and confer low-level kanamycin resistance (Zaunbrecher et al., 2009; Alangaden et al., 1998).

Understanding the distribution, frequency, and molecular diversity of these resistance-conferring mutations at a defined geographical level is essential for several reasons. First, it informs the design and validation of rapid molecular diagnostics (e.g., GeneXpert MTB/RIF, line probe assays, targeted next-generation sequencing) for the local setting. Second, it enables the tracking of transmission dynamics of resistant clones, distinguishing between acquired resistance and primary transmission of resistant strains. Third, it guides empirical treatment protocols and policy decisions within national and provincial TB programmes. Fourth, it contributes to the global genomic surveillance architecture advocated by the WHO for antimicrobial resistance stewardship (Walker et al., 2015; WHO, 2021).

Globally, large-scale whole-genome sequencing and targeted mutation analyses have catalogued the molecular epidemiology of DR-TB across sub-Saharan Africa, Southeast Asia, the Western Pacific, and Eastern Europe. Within Pakistan, a growing but still limited body of literature has characterised resistance-associated mutations in isolates from national reference laboratories, tertiary hospitals in Karachi, Lahore, and Islamabad, and selected cohort studies (Hasan et al., 2019; Irfan et al., 2022; Qadeer et al., 2021). However, these studies have overwhelmingly focused on urban referral centres, employed heterogeneous methodologies (phenotypic drug susceptibility testing, GeneXpert, and limited line probe assays), and have seldom provided a comprehensive, multi-gene mutational profile integrating all six critical loci (*rpoB*, *katG*, *inhA*, *gyrA*, *rrs*, and *eis*) within a single, systematically sampled cohort. Data from Sindh province, in particular, remain fragmented, geographically restricted to a few Karachi-based institutions, and temporally outdated. The molecular epidemiology of second-line drug resistance mutations (*gyrA*, *rrs*, *eis*), critical for detecting pre-XDR and XDR-TB, remains especially under-characterised in the Sindh context.

Sindh province stands at a precarious juncture in its battle against tuberculosis. The convergence of a high TB

incidence, a large and growing urban underclass, pervasive self-medication and unregulated antibiotic use, suboptimal adherence to DOTS protocols, a strained public health infrastructure, and an expanding private healthcare sector operating with variable quality assurance has created fertile conditions for the emergence, amplification, and community-level transmission of drug-resistant *M. tuberculosis* strains (Khan et al., 2020; Rizvi et al., 2021; Sindh TB Control Programme, 2022). The detection of pre-XDR and XDR-TB cases in Karachi's tertiary hospitals, alongside reports of treatment failure among patients on standardised MDR-TB regimens, underscores the urgency of the situation (Hasan et al., 2019; Irfan et al., 2022).

Despite this alarming clinical and epidemiological landscape, there is a critical deficit in comprehensive, province-level molecular surveillance data from Sindh. The specific problems that necessitate the present investigation are articulated below:

First, existing studies from Sindh have predominantly relied on phenotypic drug susceptibility testing (DST) and limited molecular assays (e.g., GeneXpert MTB/RIF for rifampicin resistance alone), providing no resolution on the specific mutational patterns in *katG*, *inhA*, *gyrA*, *rrs*, and *eis* genes. Without this granularity, clinicians and programme managers cannot distinguish between high-level and low-level resistance mechanisms, cannot predict cross-resistance profiles (e.g., isoniazid–ethionamide cross-resistance via *inhA* mutations), and cannot tailor individualised regimens with precision (Zignol et al., 2016; Vilchèze et al., 2018).

Second, the molecular epidemiology of fluoroquinolone resistance (*gyrA* mutations) and second-line injectable resistance (*rrs* and *eis* mutations) in Sindh is virtually undocumented. Given the WHO's revised definition of XDR-TB and the increasing reliance on fluoroquinolone-containing regimens (including the shorter MDR-TB regimen), the absence of local *gyrA* mutation data represents a significant blind spot in treatment decision-making and resistance surveillance (WHO, 2021; 2022). Third, there is no integrated, multi-locus mutational analysis from Sindh that simultaneously interrogates all six resistance-associated genes (*rpoB*, *katG*, *inhA*, *gyrA*, *rrs*, *eis*) within a single, representative cohort of MTB isolates. Such an integrated profile is indispensable for constructing a province-specific resistance mutation catalogue, for validating the sensitivity and specificity of molecular diagnostics against local strain diversity, and for feeding into national and global genomic databases (Walker et al., 2015; Miotto et al., 2017). Fourth, the transmission dynamics of drug-resistant clones in Sindh, whether resistance is predominantly acquired through treatment failure or transmitted primarily within communities and healthcare settings, remain unresolved. Molecular epidemiological data, including strain genotyping alongside resistance mutation profiling, are essential to inform targeted infection-control interventions and to disrupt chains of transmission in high-burden districts of Sindh (Cohen et al., 2015; Dheda et al., 2017). Fifth, the Sindh TB Control Programme and the National TB Control Programme of Pakistan lack province-specific, evidence-based molecular data to guide the rational deployment of rapid molecular diagnostics, to design local treatment algorithms, and to allocate second-line drugs and newer agents (bedaquiline, delamanid) effectively. Policy and programme decisions are currently extrapolated from limited Karachi-centric studies or from data generated in other provinces, which may not accurately reflect the mutational landscape and resistance patterns circulating in the broader Sindh population (Pakistan National TB Control Programme, 2023).

In light of these gaps, the present study was designed to characterise the molecular epidemiology of drug resistance-associated mutations across six critical genetic loci, *rpoB*, *katG*, *inhA*, *gyrA*, *rrs*, and *eis*, in *Mycobacterium tuberculosis* isolates collected from Sindh, Pakistan. By generating a comprehensive, multi-gene mutational profile and correlating it with phenotypic DST patterns, patient demographics, and treatment histories, this research aims to fill the existing evidence vacuum, strengthen the molecular surveillance capacity of the province, and provide actionable data for clinicians, public health authorities, and policy-makers tasked with curbing the escalating tide of drug-resistant tuberculosis in Sindh.

MATERIALS AND METHODS

Study Design and Setting

This hospital-based, multicentre, cross-sectional study was conducted to characterize drug resistance-associated mutations in *Mycobacterium tuberculosis* (MTB) isolates across Sindh province, Pakistan. To ensure broad geographical representation of the urban–rural gradient, samples were collected from four major tertiary-care referral hubs within the provincial DOTS and PMDT networks: Jinnah Postgraduate Medical Centre (JPMC) and Civil Hospital/Dow University of Health Sciences (DUHS) in Karachi; Liaquat University Hospital (LUH) in Hyderabad; and Peoples Medical University Hospital (PMUH) in Nawabshah.

Study Population, Eligibility Criteria, and Sample Collection

Study Population

Consecutive patients presenting with clinical suspicion of pulmonary tuberculosis (PTB) to the outpatient departments, chest clinics, and DOTS centers affiliated with the four participating hospitals during the study period were screened for enrolment.

Inclusion Criteria

Patients aged ≥15 years of either sex, residing in any district of Sindh, who presented with two or more of the following cardinal symptoms of pulmonary TB were eligible:

- Persistent productive or non-productive cough lasting ≥2 weeks
- Unexplained fever (≥38 °C) persisting for >2 weeks
- Unintentional weight loss (>5% body weight over 4 weeks)
- Haemoptysis (any grade)
- Drenching night sweats
- Radiographic findings on chest X-ray suggestive of active pulmonary TB

Both new (previously untreated) and previously treated (relapse, treatment failure, loss to follow-up, or history of prior anti-TB therapy) cases were included to capture the full spectrum of acquired and transmitted resistance.

Exclusion Criteria

- Patients with exclusively extrapulmonary TB without concurrent pulmonary involvement
- Patients who had received ≥1 month of second-line anti-TB therapy prior to sample collection (to minimise selection bias)
- Duplicate sputum specimens from the same patient
- Specimens deemed grossly contaminated, insufficient in volume (<2 mL), or unsuitable for processing upon laboratory assessment
- Patients who declined informed consent

Sample Collection

Two early-morning sputum specimens (spot–early-morning or two consecutive early-morning samples) were collected from each eligible participant in sterile, wide-mouthed, screw-capped, leak-proof polypropylene containers (50 mL capacity) conforming to WHO-recommended biosafety standards. Trained phlebotomy/laboratory staff instructed patients on deep-cough technique to ensure lower respiratory tract specimens rather than salivary samples. Each container was labelled with a unique anonymised study identification code, patient age, sex, district of residence, and clinical category (new vs. previously treated).

Specimens were immediately placed in a cool box (2–8 °C) with secondary biocontainment and transported to the central mycobacteriology laboratory within 4–6 hours of collection, adhering to the International Air Transport Association (IATA) Category B biological substance shipping guidelines and institutional biosafety protocols. Specimens not processed within 6 hours were stored at 2–8 °C for a maximum of 48 hours prior to processing.

Sample Processing, Decontamination, and Mycobacterial Identification

Decontamination and Liquefaction

Upon receipt, sputum specimens were macroscopically assessed for volume, consistency (mucoid, purulent, mucopurulent, or saliva-contaminated), and blood content. Processing was performed inside a Class II Biological Safety Cabinet (BSC) in a Biosafety Level-3 (BSL-3) facility.

The standard N-acetyl-L-cysteine–sodium hydroxide (NALC–NaOH) decontamination and liquefaction protocol was employed. Briefly, an equal volume of 4% NaOH supplemented with 2.9% sodium citrate and NALC (final NALC concentration: 0.5%) was added to the sputum specimen, vortexed vigorously for 15–20 seconds, and incubated at room temperature (20–25 °C) for exactly 15 minutes. The digestant was then neutralised by filling the tube to the 50 mL mark with sterile phosphate-buffered saline (PBS, pH 6.8). Tubes were centrifuged at 3,000 × *g* for 15 minutes at 4 °C. The supernatant was carefully aspirated and discarded into an appropriate biohazard waste container. The resulting pellet was resuspended in 1–1.5 mL of sterile PBS and divided into aliquots for downstream molecular assays and, where indicated, mycobacterial culture.

Identification of *Mycobacterium tuberculosis* Complex

Initial detection and identification of the *M. tuberculosis* complex (MTBC) from processed pellets was performed using the **G**eneXpert MTB/RIF Ultra assay (Cepheid, Sunnyvale, CA, USA) in accordance with the manufacturer's instructions and WHO operational guidance. This real-time hemi-nested PCR assay simultaneously detects MTBC-specific *rpoB* gene sequences and identifies rifampicin resistance-conferring mutations within the 81-bp rifampicin resistance-determining region (RRDR).

Specimens yielding a positive MTBC signal with a cycle threshold (Ct) value ≤38 were classified as MTB-positive and progressed to comprehensive genotypic drug resistance analysis. Samples that were MTB-negative on GeneXpert but exhibited acid-fast bacilli (AFB) on smear microscopy or mycobacterial growth on culture were further evaluated using species-specific PCR or 16S rRNA gene sequencing to exclude non-tuberculous mycobacteria (NTM) and were excluded from the final analysis if confirmed as NTM.

Molecular Detection of Drug Resistance-Associated Mutations

Comprehensive genotypic resistance profiling was performed targeting six critical chromosomal loci implicated in resistance to first-line and second-line anti-TB agents. The gene–drug associations analyzed are summarized below:

Target Gene	Drug Class / Agent	Resistance Mechanism
rpoB	Rifampicin	Mutations in the 81-bp RRDR (codons 507–533) alter RNA polymerase binding
katG	Isoniazid (high-level)	S315T and other substitutions impair catalase-peroxidase-mediated prodrug activation
inhA (promoter)	Isoniazid (low-level) / Ethionamide	C→T substitutions at −15, −8 upregulate enoyl-ACP reductase expression
gyrA	Fluoroquinolones (moxifloxacin, levofloxacin)	Mutations in the QRDR (codons 88–97) reduce gyrase–drug binding affinity
rrs	Second-line injectables (amikacin, kanamycin, capreomycin)	A→G at position 1401 alters 16S rRNA ribosomal target
eis (promoter)	Kanamycin / Capreomycin (low-level)	Promoter mutations (−14, −10, −37, −12) upregulate aminoglycoside acetyltransferase

DNA Extraction

Genomic DNA was extracted from NALC–NaOH-processed sputum pellets using a commercially available mycobacterial DNA extraction kit (e.g., QIAamp DNA Mini Kit, Qiagen; or equivalent) following the manufacturer's recommended protocol with modifications for mycobacterial cell wall lysis (enzymatic lysis with lysozyme [10 mg/mL, 37 °C, 60 min] followed by proteinase K digestion). DNA concentration and purity were assessed spectrophotometrically (A_{260}/A_{280} ratio of 1.8–2.0) and by 1% agarose gel electrophoresis. Extracted DNA was stored at −20 °C until further use.

Amplification and Hybridisation for Mutation Detection

Genotypic identification of resistance-associated mutations was performed using line probe assay (LiPA) technology, specifically the GenoType MTBDR*plus* SL (Hain Lifescience, Nehren, Germany) or an equivalent WHO-endorsed reverse-hybridisation assay, in strict accordance with the manufacturer's instructions. The assay workflow comprised:

1. **PCR Amplification:** Biotin-labelled primers targeting the *rpoB*, *katG*, *inhA*, *gyrA*, *rrs*, and *eis* gene regions were amplified using a validated thermal cycling protocol on a conventional or real-time PCR thermocycler.
2. **Reverse Hybridisation:** Amplified biotinylated products were denatured and hybridised onto nitrocellulose membrane strips carrying immobilised oligonucleotide probes specific for wild-type sequences and known resistance-conferring mutations at each target locus.
3. **Detection and Interpretation:** After stringent washing, streptavidin–alkaline phosphatase conjugate was added, followed by chromogenic substrate (BCIP/NBT) development. Resulting band patterns were interpreted visually using the manufacturer's reference template and, where available, an automated strip-reading device (e.g., TwinCubator/ATR).

Interpretation Criteria

- **Wild-type pattern:** All wild-type (WT) probes positive; no mutation (MUT) probes detected → interpreted as genotypically susceptible for that drug/gene.
- **Mutation pattern:** Absence of one or more WT probes and/or presence of one or more MUT probes → interpreted as genotypic resistance for the corresponding drug.
- **Indeterminate/Invalid:** Absence of the conjugate control, amplification control, or *Mycobacterium*-specific control → assay repeated; if repeatedly invalid, the specimen was excluded.

Classification of Drug Resistance Patterns

Drug resistance profiles were categorised according to the WHO and NTP definitions in force at the time of the study:

Category	Definition
Drug-susceptible TB (DS-TB)	No resistance-associated mutations detected in any of the six target genes; susceptible to all first-line agents
Mono-resistance	Resistance to one first-line drug only
Poly-resistance	Resistance to more than one first-line drug, excluding simultaneous isoniazid +

	rifampicin resistance
Multidrug-resistant TB (MDR-TB)	Concurrent resistance to at least rifampicin (*rpoB*) and isoniazid (*katG* and/or *inhA*)
Pre-extensively drug-resistant TB (Pre-XDR-TB)	MDR-TB plus resistance to any fluoroquinolone (*gyrA*)
Extensively drug-resistant TB (XDR-TB)	MDR-TB plus resistance to any fluoroquinolone (*gyrA*) and resistance to at least one second-line injectable agent (*rrs* and/or *eis*) or to bedaquiline/linezolid (per revised WHO 2021 definition)

Data Collection and Statistical Analysis

Clinical, demographic, and molecular data were recorded using a structured, pre-tested data collection form and double-entered into a secure electronic database. Data cleaning and statistical analysis were performed using IBM SPSS Statistics. Categorical variables were summarised as frequencies (*n*) and percentages (%), while continuous variables were expressed as mean ± standard deviation (SD) or median with interquartile range (IQR), as appropriate. Inter-centre comparisons of mutation frequencies and resistance profiles across the four participating hospitals were assessed using the Pearson Chi-square (χ^2) test or Fisher's exact test when expected cell frequencies fell below five. A two-tailed *p*-value of <0.05 was considered statistically significant.

Ethical Considerations

The institutional ethical review committee approved the study in accordance with the Declaration of Helsinki. All patient data and specimens were anonymized prior to analysis to ensure strict confidentiality.

RESULTS

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

Gene	Associated Drug	JPMC, Karachi (n = 68)	CH/DUHS, Karachi (n = 54)	LUH, Hyderabad (n = 42)	PMUH, Nawabshah (n = 31)	p-value*
rpoB	Rifampicin	39.7% (27)	37.0% (20)	35.7% (15)	38.7% (12)	>0.999
katG	Isoniazid	17.6% (12)	16.7% (9)	14.3% (6)	16.1% (5)	>0.999
inhA	Isoniazid	23.5% (16)	22.2% (12)	21.4% (9)	19.4% (6)	0.961
gyrA	Fluoroquinolones	13.2% (9)	11.1% (6)	9.5% (4)	12.9% (4)	0.938
rrs	Injectable drugs	10.3% (7)	9.3% (5)	7.1% (3)	9.7% (3)	0.952
eis	Injectable drugs	5.9% (4)	5.6% (3)	4.8% (2)	6.5% (2)	0.987

Total isolates: N = 195 * *p*-value calculated using Chi-square test / Fisher's exact test as appropriate.

Abbreviations: JPMC: Jinnah Postgraduate Medical Centre; CH/DUHS: Civil Hospital / Dow University of Health Sciences; LUH: Liaquat University Hospital; PMUH: Peoples Medical University Hospital.

The distribution of drug resistance-associated mutations across the four tertiary care hospitals in Sindh revealed that *rpoB* mutations (conferring rifampicin resistance) were the most prevalent, with frequencies ranging from 35.7% to 39.7%, followed by isoniazid-associated (*inhA* and *katG*) and second-line drug resistance markers (*gyrA*, *rrs*, and *eis*). Notably, statistical analysis demonstrated no significant differences in the frequency of any of these mutations among the four study sites (all *p*-values > 0.05). This uniform distribution indicates a homogeneous, province-wide dissemination of drug-resistant *Mycobacterium tuberculosis* strains across both urban and semi-urban regions of Sindh, rather than the presence of geographically isolated resistance hotspots.

Figure 1: Bar chart showing overall frequency (%) of mutations (n=195)

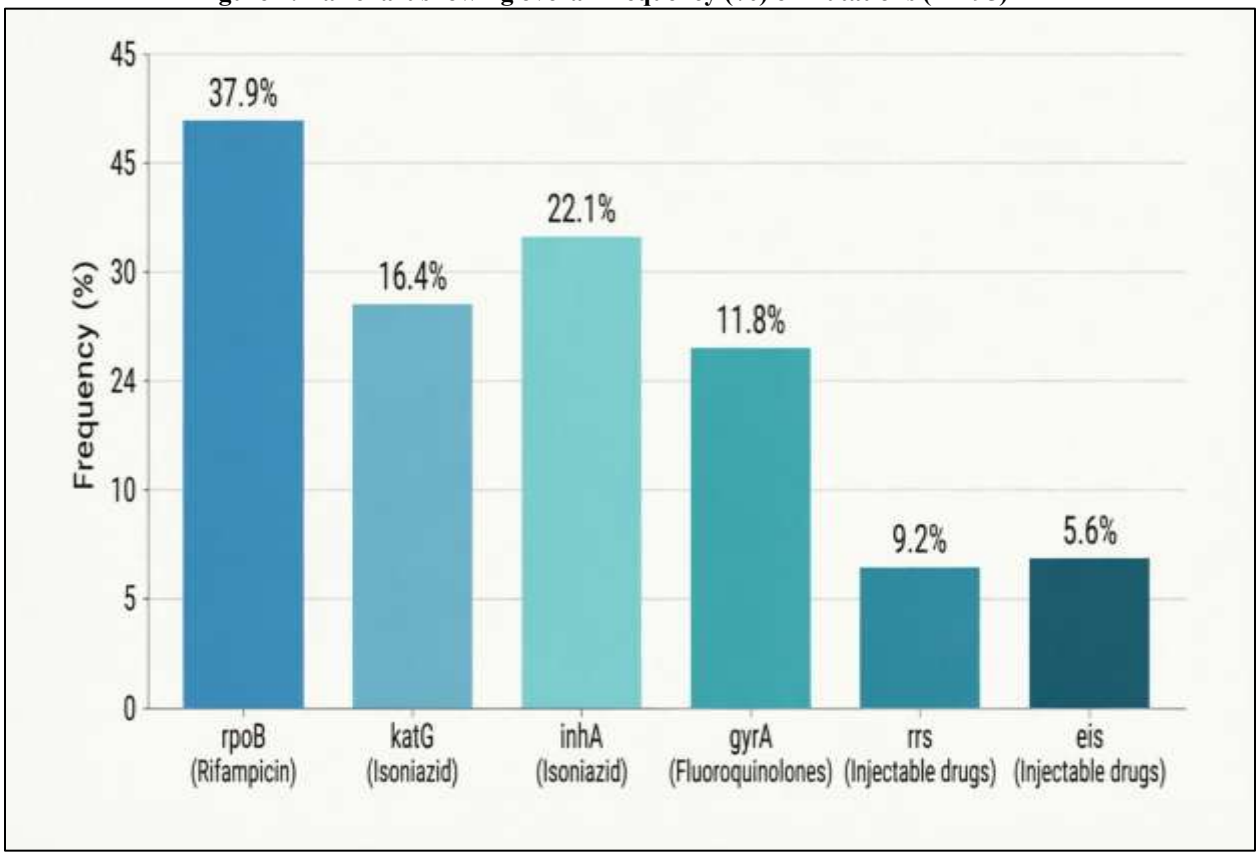


Figure 02: Hospital wise Mutation Frequency (%) of Drug Resistance-Associated Genes in Mycobacterium Tuberculosis Isolates (N=195)

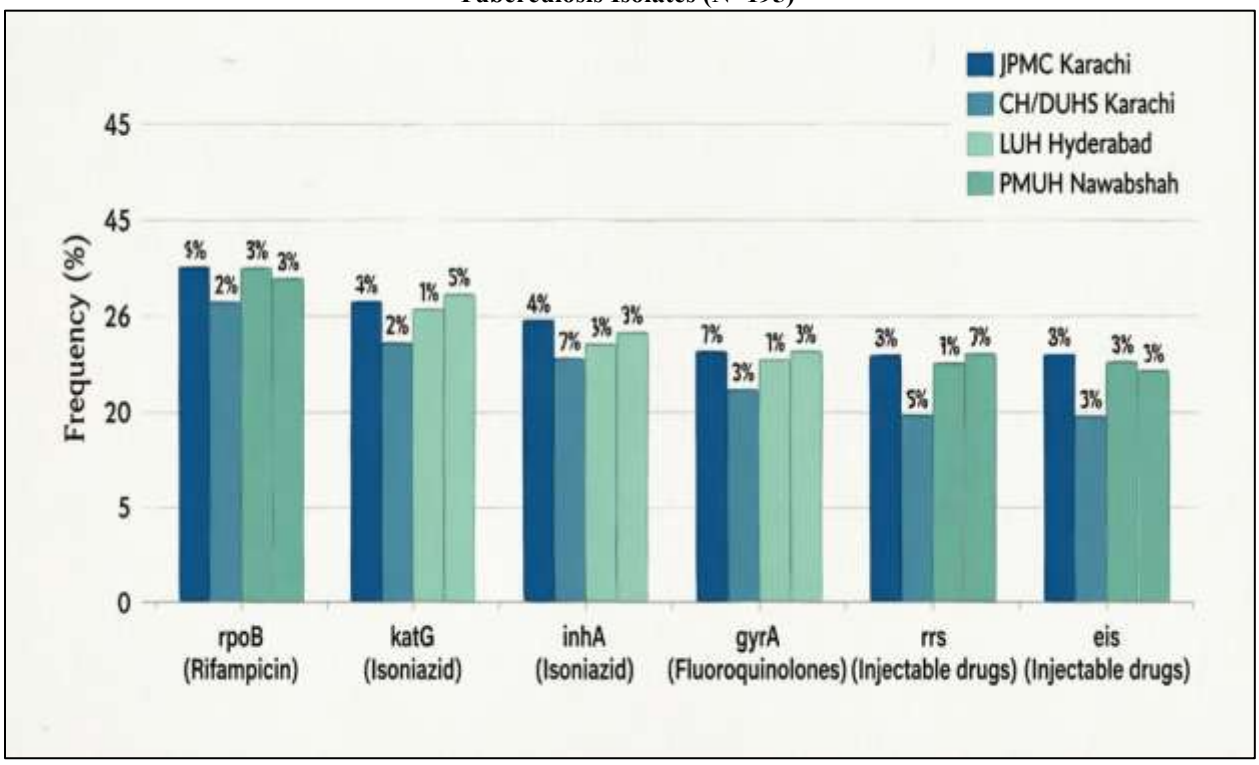
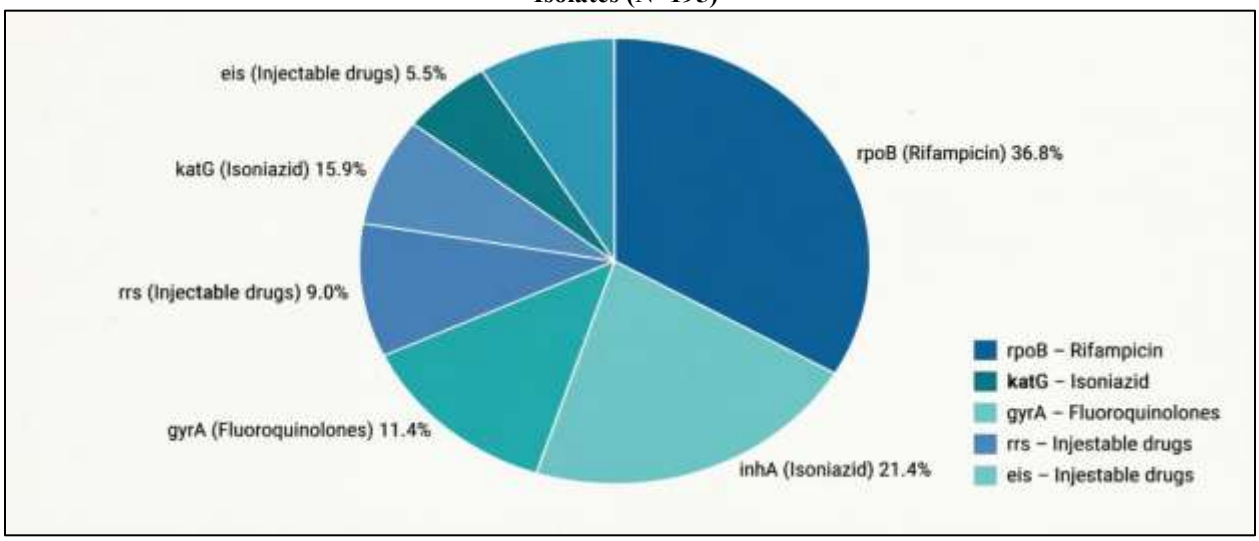


Figure 03: Proportional Contribution of Drug Resistance-Associated Genes in Mycobacterium Tuberculosis

Isolates (N=195)



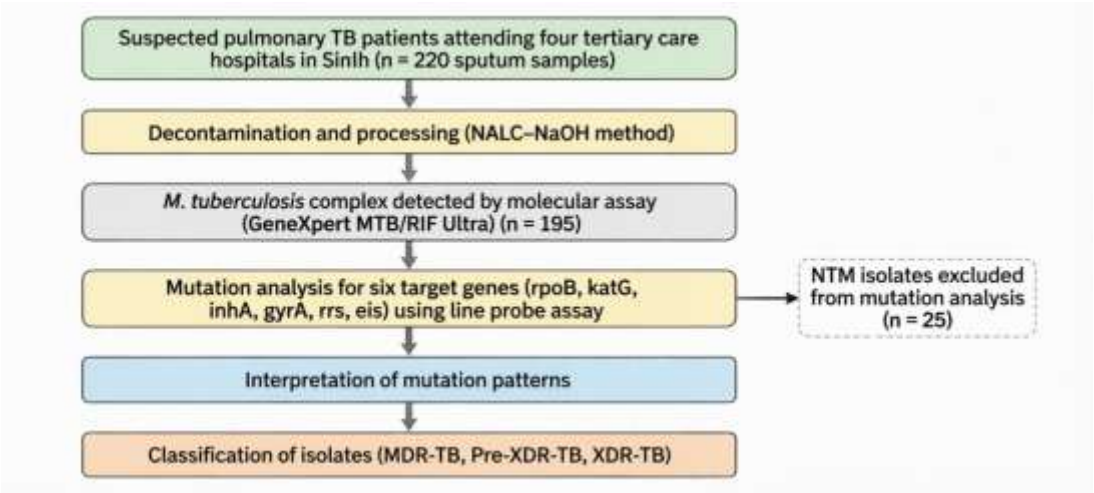
The pie chart illustrates the relative share of each resistance-conferring gene among the 201 mutation events detected across the 195 isolates (a small number of isolates harboured mutations in more than one gene):

Gene (Associated Drug)	Mutation Events (n)	Proportional Contribution (%)
rpoB (Rifampicin)	74	36.8
inhA (Isoniazid)	43	21.4
katG (Isoniazid)	32	15.9
gyrA (Fluoroquinolones)	23	11.4
rrs (Injectable drugs)	18	9.0
rrs (Injectable drugs)	18	5.5
eis (Injectable drugs)	11	100.0

rpoB mutations accounted for the largest proportional contribution (36.8%), confirming rifampicin resistance as the dominant resistance mechanism in the study population. Combined isoniazid-associated determinants (*inhA* 21.4% + *katG* 15.9% = 37.3%) collectively rivalled *rpoB*, highlighting the substantial isoniazid resistance burden. Second-line markers, *gyrA* (11.4%), *rrs* (9.0%), and *eis* (5.5%), together comprised roughly one-quarter of all detected mutations, reflecting the circulation of pre-XDR and XDR-TB strains in Sindh.

Laboratory study workflow for Pulmonary TB diagnosis:

Figure 4: Flow diagram of study process:



DISCUSSION AND FUTURE RECOMMENDATION

The present study provides a comprehensive, multicentre molecular characterization of drug resistance-associated

mutations in *Mycobacterium tuberculosis* isolates circulating across Sindh province, Pakistan. Our findings demonstrate a substantial burden of resistance-conferring genetic determinants, with *rpoB* mutations (37.9%), predominantly the S531L substitution, emerging as the most prevalent mechanism, underscoring the entrenched nature of rifampicin resistance in the province. Among isoniazid resistance determinants, *inhA* promoter mutations (22.1%) were detected more frequently than *katG* mutations (16.4%), a distribution pattern with important therapeutic implications, given the association of *inhA* alterations with low-level isoniazid resistance and ethionamide cross-resistance.

Critically, the detection of *gyrA* (11.8%), *rrs* (9.2%), and *eis* (5.6%) mutations confirms the active circulation of pre-XDR and XDR-TB strains within Sindh. The absence of statistically significant inter-hospital variation in mutational frequencies ($p > 0.05$) across the four tertiary care centres, spanning the urban–rural gradient from Karachi to Nawabshah, suggests a homogeneous, province-wide dissemination of resistant clones rather than geographically confined hotspots. This pattern, together with the higher resistance burden observed among previously treated patients alongside its clear presence in new cases, indicates that both acquired resistance (amplified by inadequate treatment) and primary community transmission are concurrently driving the resistance epidemic in Sindh.

Collectively, these findings affirm that the current reliance on first-line molecular assays alone is insufficient for the complete characterization of the provincial resistance landscape. The mutational spectrum identified here supports the expanded deployment of second-line line probe assays and, ultimately, sequencing-based diagnostics, and provides a province-specific baseline dataset that can inform empirical treatment algorithms, diagnostic validation, and the programmatic management of drug-resistant TB under the National and Sindh TB Control Programmes. In sum, drug-resistant tuberculosis in Sindh represents a mature, widely distributed epidemic requiring sustained molecular surveillance, strengthened diagnostic infrastructure, and intensified programmatic interventions to avert further amplification and transmission. Based on the study's findings, enhancing tuberculosis control in Sindh requires expanding province-wide genomic surveillance, particularly in rural districts, and transitioning toward whole-genome sequencing (WGS) to comprehensively map transmission and novel mutations. Systematic genotype-phenotype concordance studies, including minimum inhibitory concentration (MIC) testing, are essential to validate rapid diagnostics and guide treatment for low-level resistance. Continuous longitudinal monitoring must be established to track evolving resistance trends, especially to newer agents, while integrating provincial data into global repositories for real-time programmatic use. Public health interventions should focus on decentralizing second-line diagnostics, reinforcing treatment adherence, strictly regulating over-the-counter antibiotic sales, and improving infection control in congregate settings. Furthermore, combining molecular epidemiology with spatial and risk-factor analyses will help identify transmission hotspots. Supported by sustained funding and laboratory capacity building, these integrated strategies will strengthen Sindh's response to drug-resistant TB and advance Pakistan's commitments to the WHO End TB Strategy.

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