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DISTRIBUTION OF RIFAMPICIN- AND ISONIAZID-RESISTANCE-ASSOCIATED MUTATIONS IN PEDIATRIC MULTIDRUG-RESISTANT TUBERCULOSIS IN KHYBER PAKHTUNKHWA

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ABSTRACT

Background: Multidrug-resistant tuberculosis (MDR-TB) remains a major challenge in children, and the molecular patterns underlying rifampicin (RIF) and isoniazid (INH) resistance may vary geographically. Data on resistance-associated mutations among pediatric MDR-TB patients in Khyber Pakhtunkhwa (KP), Pakistan, remain limited.

Objective: To determine the frequency and distribution of RIF- and INH-resistance-associated mutation patterns, including individual and combined mutations in the *rpoB*, *katG*, and *inhA* genes, among pediatric patients with MDR-TB in KP, Pakistan.

Methodology: This study included 266 pediatric MDR-TB patients. Drug resistance-associated mutation patterns were assessed using the GenoType MTBDRplus line probe assay. The assay was used to identify mutations and wild-type probe patterns within the *rpoB*, *katG*, and *inhA* regions. Individual and combined mutation patterns were summarized using frequencies and percentages, and molecular findings were compared with phenotypic drug susceptibility testing results.

Results: RIF resistance was detected by LPA in 211 (79.32%) isolates, while INH resistance was detected in 225 (84.58%). Among RIF-resistant isolates, 113 (53.55%) showed loss of one or more *rpoB* wild-type probes without a corresponding detectable mutant probe, consistent with an uncharacterized resistance-associated change within the targeted region. Among the 98 isolates with identifiable *rpoB* mutant patterns, S531L was the most frequent single mutation (37.75%), followed by D516V (19.39%) and H526D (7.14%). Combined mutations included D516V+S531L (7.14%) and H526D+S531L (3.06%). For INH resistance, *katG*-associated patterns were more frequent than *inhA*-associated patterns.

Conclusion: Pediatric MDR-TB in KP demonstrates diverse RIF and INH-resistance-associated mutation patterns, with *rpoB* S531L and *katG*-associated resistance being prominent. The substantial proportion of unresolved LPA patterns highlights the need for sequencing-based studies to characterize unrecognized resistance-associated mutations.

KEYWORDS: Tuberculosis; Multidrug Tuberculosis; Mutation; rpoB Gene; katG Gene; inhA Gene; Khyber Pakhtunkhwa.

INTRODUCTION

Despite advances that have been made in the diagnosis, treatment, and prevention of tuberculosis (TB), the disease continues to pose a significant global public health threat. This issue is particularly acute among children and adolescents, who experience high rates of morbidity and mortality and frequently encounter diagnostic delays. The emergence of drug-resistant TB (DR-TB) further complicates control efforts by limiting effective treatment options and facilitating ongoing transmission. The World Health Organization (WHO) defines multidrug-resistant TB (MDR-TB) as resistance to at least rifampicin (RIF) and isoniazid (INH), which are two essential first-line drugs. In 2023, approximately 400,000 individuals developed MDR/RR-TB, underscoring the persistent challenge of drug resistance.[1]

Children affected by MDR-TB are especially vulnerable. Diagnosis in children is challenging because of low bacterial loads and difficulty in obtaining appropriate clinical specimens, which often delays confirmation of drug resistance and treatment initiation. The WHO emphasizes the importance of rapid diagnosis and effective management of MDR/RR-TB in pediatric populations, and has broadened treatment options for these patients.[2] Nevertheless, elucidating the molecular mechanisms underlying resistance remains essential to improve diagnostic accuracy and uniform treatment strategies, particularly in regions with a high disease burden.

The primary cause of resistance to anti-TB drugs is genetic changes that alter drug targets or pathways involved in drug activation and susceptibility. Most rifampicin resistance is associated with mutations in the rifampicin resistance-determining region (RRDR) of the rpoB gene, which encodes the β-subunit of bacterial RNA

polymerase. Frequently observed resistance-associated substitutions include mutations at codons D516, H526, and S531.[3] Isoniazid resistance is genetically more heterogeneous and is frequently associated with alterations in the katG gene, particularly mutations at codon S315, and mutations in the inhA promoter region. The distribution of these mutations varies widely across geographic regions and populations.[4]

Detecting resistance-associated mutations has become an important component of rapid TB diagnostics. Molecular assays can identify resistance-associated genetic alterations much more rapidly than conventional culture-based drug susceptibility testing, enabling earlier detection of MDR-TB and more timely initiation of appropriate treatment.[5] The GenoType MTBDRplus line probe assay (LPA) targets the rpoB, katG, and inhA regions and detects the common mutations associated with RIF and INH resistance. In addition to specific mutant probes, the loss of hybridization of the wild-type probe may indicate the presence of a mutation in the target region that is not specifically represented by a mutation probe. Therefore, LPA results can provide valuable information not only on the existence of resistance but also on the distribution of frequently recognized resistance-associated mutation patterns.[6]

The epidemiological distribution of resistance-conferring mutations varies across populations. The prevalence of individual mutations in the genes rpoB, katG, and inhA has been shown to vary significantly across studies in different geographic locations. The two most commonly encountered mutations are S531L in rpoB and S315T in katG; however, the importance of these genes, along with other mutations, varies by region. A study using GenoType MTBDRplus and MTBDRsl tests found rpoB S531L and inhA promoter C15T to be the dominant mutations in drug-resistant isolates.[6] Similarly, recent molecular investigations have shown that LPA can detect varied mutation patterns.[7]

Pakistan continues to be one of the countries where a large burden of TB, as well as drug-resistant TB, persists.[8] Molecular studies of TB isolates from Pakistan have revealed geographical relevance in mutation patterns conferring resistance. A previous molecular study in the Khyber Pakhtunkhwa region of Pakistan found a high prevalence of rpoB mutations conferring rifampicin resistance, with mutations in the 81 bp RRDR forming an important molecular basis for resistance.[9] More recent molecular analyses of isoniazid resistance have identified mutations in the katG gene at the S315T position and in the inhA promoter as key resistance determinants.[10] However, most molecular evidence from Pakistan is based on general TB isolates or adults with TB.

Knowing the molecular profile of resistance in pediatric MDR-TB improves understanding of how resistance occurs and how this might affect result interpretation. It can also help explain how resistant *M. tuberculosis* strains are transmitted in the community. Another benefit is that identifying mutant probes in the wild-type probe and in the combined mutations improves understanding of the molecular profile of resistance.

Although molecular diagnostic testing is available in Pakistan, systematic investigation of rifampicin- and isoniazid-resistance-associated mutations in children with MDR-TB from Khyber Pakhtunkhwa remains needed. This study sought to identify the frequency and distribution of rifampicin- and isoniazid-resistance-associated mutation patterns, including individual and combined mutations in the rpoB, katG, and inhA genes, in pediatric MDR-TB patients from Khyber Pakhtunkhwa. By characterizing regional molecular resistance patterns in pediatric MDR-TB patients, this study could improve understanding of regional resistance patterns.

Objective: To determine the frequency and distribution of RIF- and INH-resistance-associated mutation patterns, including individual and combined mutations in the *rpoB*, *katG*, and *inhA* genes, among pediatric patients with MDR-TB in KP, Pakistan.

METHODOLOGY

This multi-center prospective cohort study was conducted in Programmatic Management of Drug-Resistant TB (PMDT) Units in Khyber Pakhtunkhwa (KP). The present study included all children with resistant tuberculosis. Strict inclusion and exclusion criteria were followed for the selection of cases. Inclusion criteria included children aged ≤14 years with confirmed MDR-TB, and all such cases were excluded from the study if they were older than 14 years or had sensitive TB.

The sample size was based on the estimated incidence of drug-resistant TB in children using Daniel's sample size calculation formula (Daniel & Wayne, 1995) with Z = 1.96 for a 95% confidence interval, with 21.2% (4.2% in new and 16.0% in retreatment cases)[11] as the prevalence rate of MDR-TB with a 5% margin of error. The estimated sample size was 266. For selection of these cases, a proportionate stratified sampling technique was used. All patients meeting the inclusion criteria were interviewed at each monthly follow-up visit for treatment at the study center. All study cases were followed up till the end of the study duration.

All TB suspect cases who visited PMDT units were screened for MTB using the GeneXpert® MTB/RIF assay according to the manufacturer's instructions (Cepheid, 2023).[12] Following this, if any case was declared positive for MTB and RRD on GeneXpert, another 5–10 mL "morning" sputum sample was collected from each patient at each PMDT Unit using a properly labeled disposable Falcon tube. All of the collected sputum samples were kept in a cold chain and transported to the main PRL Laboratory for culture and Drug susceptibility testing.[13]

Line probe assay (LPA) was performed using the GenoType® MTBDRplus Version 2.0 kit (Hain Lifescience GmbH, Nehren, Germany) according to the manufacturer's instructions, to detect mutations associated with resistance to rifampicin (RIF) and isoniazid (INH) in *Mycobacterium tuberculosis* complex (MTBC) isolates.

DNA was isolated from the decontaminated and concentrated samples of sputum sediments through the use of the GenoLyse® kit (Hain Lifescience GmbH, Nehren, Germany). Specifically, 500 µL of the treated sediment was

first centrifuged and then suspended in lysis buffer, after which neutralizing buffer was added. After incubating the mixture at 95°C for 10 minutes, it was centrifuged again, and the supernatant, containing the extracted DNA, was harvested.

The isolated DNA sample was amplified using a thermal cycler with specific primers for the rpoB gene (rifampicin resistance-determining region), the katG gene, and the inhA promoter region. The PCR reaction contained 35 µL of primer nucleotide mixture, 10 µL of Taq polymerase–PCR buffer, and 5 µL of DNA.

The amplified products were tested by reverse hybridization using a Twin Cubator®. The biotinylated amplified products were hybridized to nitrocellulose membranes with the WT and MUT oligonucleotide probes immobilized on them. After washing under stringent conditions, a chromogenic reaction was performed using the streptavidin-alkaline phosphatase conjugate and a substrate.

Detection of bands in the conjugate control region, amplification control region, and locus control regions (rpoB, katG, and inhA) was required for a valid test result. For drug susceptibility, detecting all wild-type bands without detecting mutant bands indicated susceptibility to that drug. Conversely, the absence of some or all wild-type bands, along with the presence of a mutant band in a particular gene, indicated resistance to that drug.

For the *rpoB* gene, we analyzed mutations at codons D516V (MUT1), H526Y (MUT2A), H526D (MUT2B), and S531L (MUT3). For the *katG* gene, mutations at codons S315T1 (MUT1) and S315T2 (MUT2) were detected. For the *inhA* gene, mutations at codons C15T (MUT1), A16G (MUT2), T8C (MUT3A), and T8A (MUT3B) in the promoter region were identified. The assay could also detect unknown mutations, represented by the deletion of wild-type bands without a corresponding mutant band.

Data were collected from enrolled pediatric MDR-TB patients using a structured data collection form. Demographic and clinical information, along with laboratory findings and line probe assay (LPA) results, were recorded from the available patient records. Genotypic resistance patterns detected by the GenoType MTBDRplus assay were documented for the *rpoB*, *katG*, and *inhA* genes, including wild-type probe patterns, identifiable mutant patterns, and combined mutation patterns.

The data were entered, coded, and analyzed using IBM SPSS Statistics version 27. Continuous variables were summarized using mean ± standard deviation or median with interquartile range, as appropriate, while categorical variables were presented as frequencies and percentages. The frequency and distribution of individual and combined rifampicin- and isoniazid-resistance-associated mutation patterns were calculated. The findings were presented descriptively using tables and percentages.

RESULTS

The present study included 266 children with Multidrug-Resistant Tuberculosis (MDR-TB), of whom 176 (66.2%) were female, and 90 (33.8%) were male. Participants were nearly evenly distributed between rural and urban areas. The present cohort predominantly comprises adolescents, with 226 (85.0%) belonging to the age group 10 - 14 years. The fewest cases were in the age group less than five years. The mean age was 12.09 (±2.294), with a median of 13 years. Among the study cases, most participants (206, 77.4%) had a prior history of tuberculosis (TB) disease. Among 206 patients previously treated for TB, 150 (56.4%) children experienced treatment failure. Only 47 (16.7%) children achieved a successful treatment outcome (Table 1).

Table 1: Baseline Characteristics of Study Participants

Variables		Frequency	Percent (%)
Gender	Male	90	33.8
	Female	176	66.2
Area of residence	Rural	132	49.6
	Urban	134	50.4
Age Group (Year)	< 5	12	4.5
	5 – 9	28	10.5
	10 – 14	226	85.0
Tuberculosis History	Yes	206	77.4
	No	60	22.6
Previous TB Treatment Outcome	Cured	16	6.0
	Completed	31	11.7
	Failure	150	56.4
	Loss to follow up	9	3.4
	Not treated previously	60	22.6
Baseline Weight (Kg)	Normal	94	35.3
	Below Normal	172	64.7

Rifampicin Detection	RRD Detected	257	96.6
	RRD Not Detected	9	3.4

Results showed that 218 (81.9%) isolates were resistant to Isoniazid (INH). Resistance to Rifampicin (RMP) was 72.9%, and 117 (44.0%) isolates showed resistance to Ethambutol (44.0%), while 8 (3.0%) isolates showed resistance to Kanamycin (3.0%) and 7 (2.6%) isolates showed resistance to Capreomycin (Figure 1).

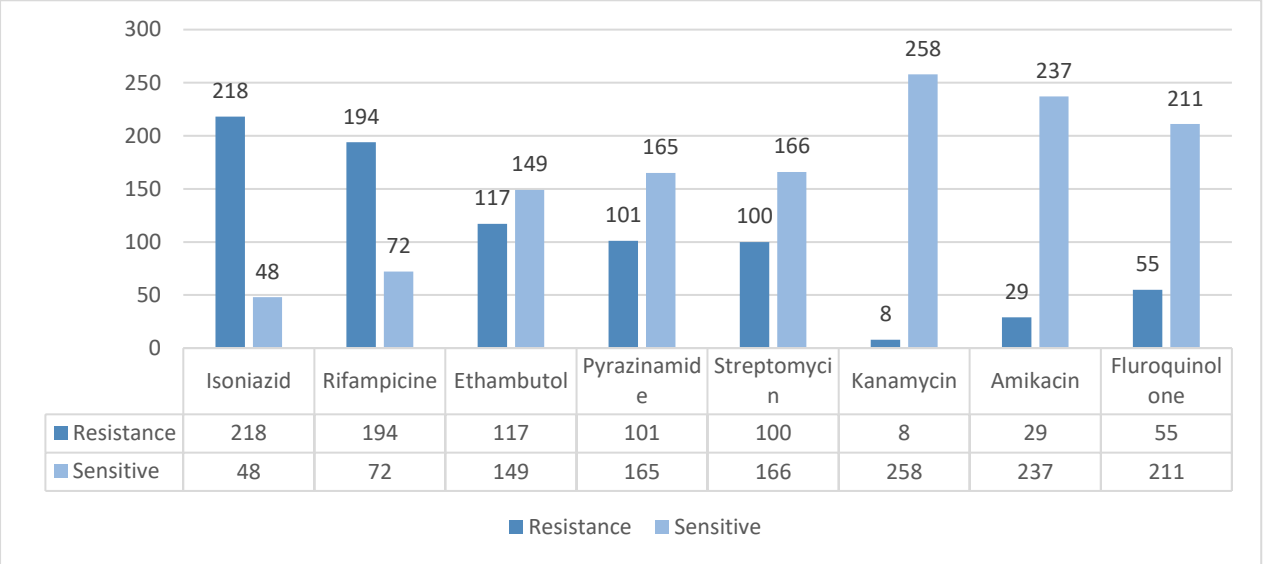

Figure 1: Drug Resistance Profiles of strains isolated from study cases

Comparison of Rifampicin and Isoniazid on DST and LPA showed that 72.9% strains showed resistance in DST and 79.32% on LPA, and the difference was 6.3% whereas resistance to isoniazid (inh A, kat G) was 81.9% on DST and 84.58% on LPA (Table 2).

Table 2: Comparison of DST and LPA

MDR Cases	DST	LPA	Difference's
Rifampicin (rpoB)	194 (72.9%)	211 (79.32%)	17 (6.3%)
Isoniazid (InhA, katG)	218 (81.9%)	225 (84.58%)	7 (2.63%)
Total	**412**	**436**	**24 (9.02%)**

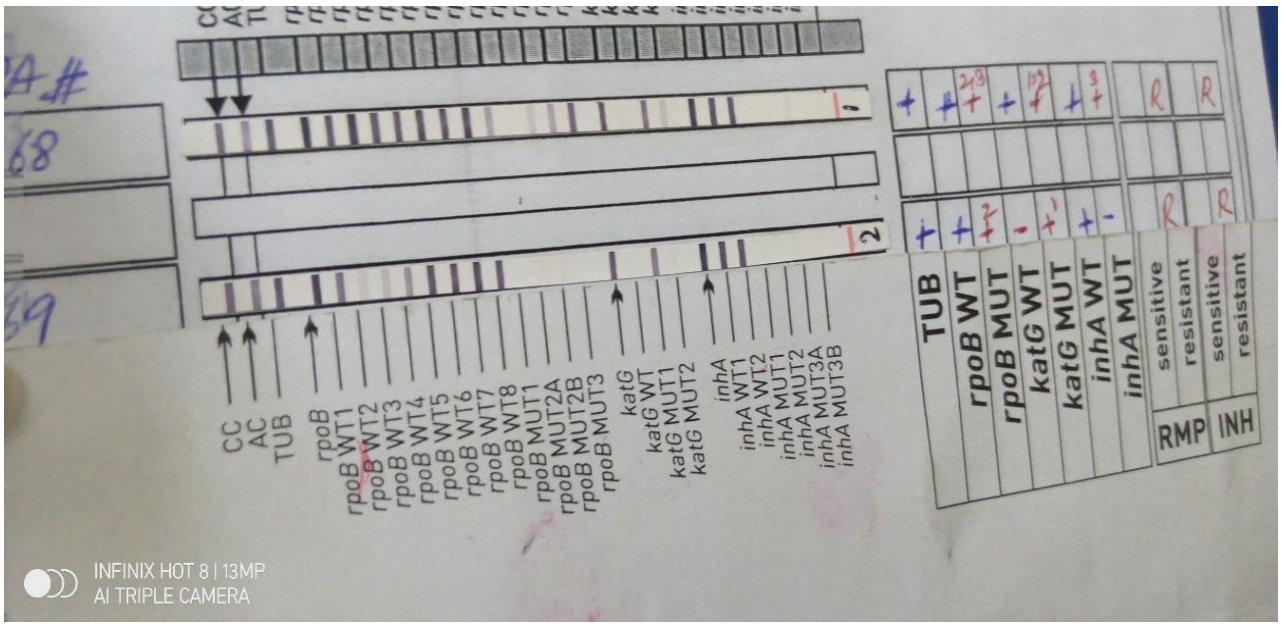

Figure 2: Mutation showing resistance to different Genes

Among the 211 rifampicin-resistant isolates identified by LPA, 113 (53.55%) showed loss of one or more rpoB wild-type probes without a corresponding detectable mutant probe, consistent with the presence of an uncharacterized resistance-associated mutation within the targeted rpoB region. Among these isolates, the most frequently observed pattern was rpoWT8 (530–533), detected in 47 (41.60%) isolates. The combination of rpoWT3 (510–517) and rpoWT4 (513–519) was observed in 10 (8.84%) isolates, while rpoWT2 (510–513) combined with rpoWT3 (510–517) was detected in 7 (6.20%) isolates. In contrast, 98 (46.45%) isolates demonstrated at least one identifiable rpoB resistance-associated mutant pattern. Among these, the most prevalent single mutation was rpoMUT3 (S531L), detected in 37 (37.75%) isolates, followed by rpoMUT1 (D516V) in 19 (19.39%) isolates and rpoMUT2b (H526D) in 7 (7.14%) isolates. Combined mutations were less frequent: rpoMUT1 (D516V) + rpoMUT3 (S531L) was detected in 7 (7.14%) isolates, whereas rpoMUT2b (H526D) + rpoMUT3 (S531L) was observed in 3 (3.06%) isolates. No isolates in this study cohort showed three simultaneous rpoB mutations (Table 3).

Table 3: Distribution of Wild-Type and Mutant Probe Patterns for rpoB Regions Detected by Line Probe Assay

Mutation All wild type Missing (R)	**Gene Region or Mutation**	**Frequency (%)** **113 (53.55%)**
rpoWT3	510-517	19 (16.81)
rpoWT7	526-529	14 (12.4)
rpoWT8	530-533	47 (41.60)
rpoWT2, rpoWT3	510-513, 510-517	07 (6.20)
rpoWT3, rpoWT4	510-517, 513-519	10 (8.84)
rpoWT2, rpoWT8	510-513, 530-533	04 (3.53)
rpoWT7, rpoWT8	526-529, 530-533	08 (7.10)
rpoWT2, rpoWT3, rpoWT4	510-513, 510-517, 513-519	04 (3.53)
rpo Gene Band		**Frequency (%)** **(98 (46.45%))**
rpoMUT1	D516V	19 (19.4)
rpoMUT3	S531L	37 (37.75)
rpoMUT2b	H526D	07 (7.14)
rpoMUT2a	H526Y	18 (18.37)
rpoMUT2a, rpoMUT3	H526Y, S531L	03 (3.06)
rpoMUT1, rpoMUT2b	D516V, H526D	04 (4.08)
rpoMUT1, rpoMUT3	D516V, S531L	07 (7.14)
rpoMUT2b, rpoMUT3	H526D, S531L	03 (3.06)

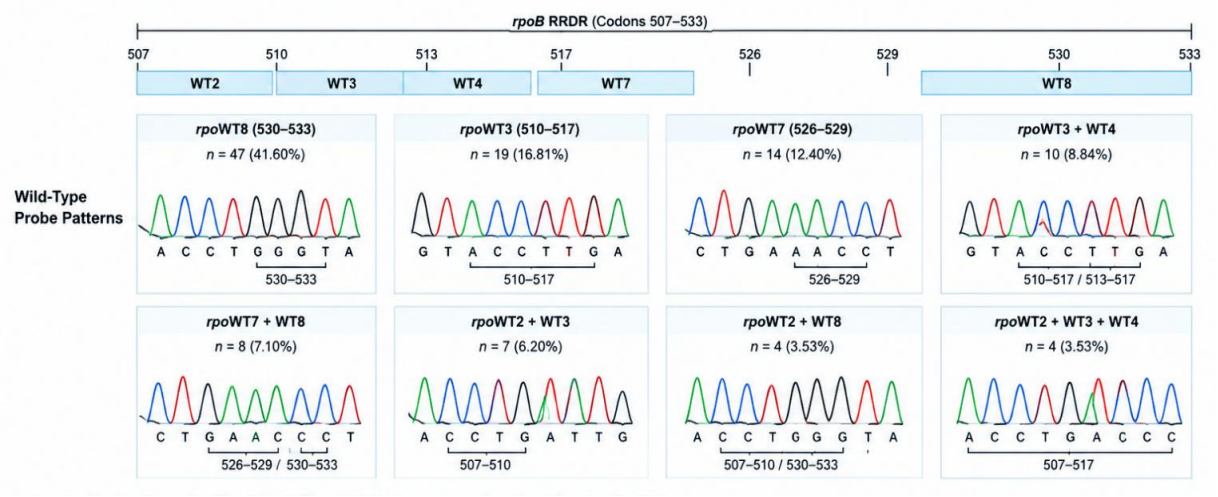

Figure 3. Distribution of rpoB Wild-Type Probe Patterns among Rifampicin-Resistant Isolates

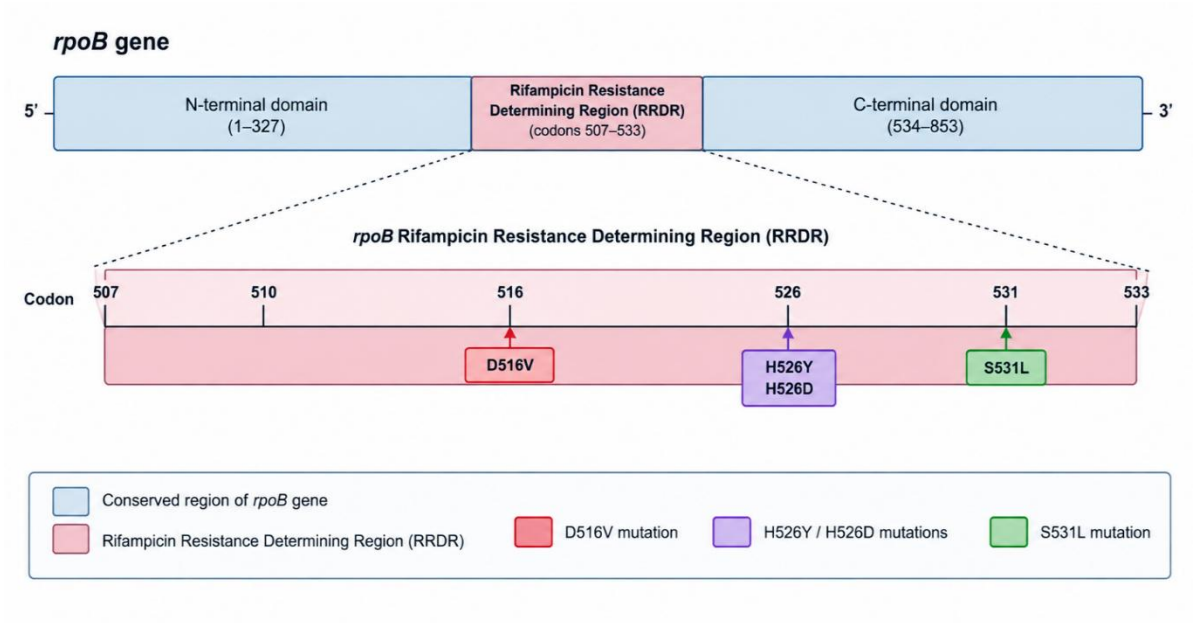

Figure 4: Mutation map of the rpoB RRDR

Figure 4 shows the distribution and location of mutation patterns detected within the rifampicin resistance-determining region (RRDR) of the *rpoB* gene. Among the mutations, the predominant mutation pattern was rpoMUT3 (S531L), which was identified in 37 (37.75%) mutant isolates, followed by rpoMUT1 (D516V) in 19 (19.39%) isolates and rpoMUT2a (H526Y) in 18 (18.37%) isolates. Single H526D mutations were less frequent, occurring in 7 (7.14%) isolates. Combined mutation patterns were uncommon, with D516V + S531L representing the most frequent dual mutation pattern (7.14%), followed by D516V + H526D (4.08%), H526Y + S531L (3.06%), and H526D + S531L (3.06%). No isolate exhibited three concurrent *rpoB* mutations.

In 225 resistant samples of Isoniazid (INH) resistance found on LPA, 91 are *inhA,* and 134 are *katG* gene resistance. Among the 91 *inhA*-positive samples, 59 (64.84%) exhibited mutant-type patterns (MUT1, MUT2, MUT3A, or MUT3B), indicating the presence of resistance-associated mutations. The remaining 32 samples (35.16%) showed wild-type patterns (WT1, WT2) with no detectable mutations in the targeted *inhA* regions. On the other hand, among the 134 *katG*-positive samples, 65 (48.50%) carried mutant-type alleles (MUT1 or MUT2), while 69 (51.49%) displayed the wild-type pattern (WT1) (Table 4).

Table 4: Distribution of Wild-Type and Mutant Probe Patterns in inhA and katG Genes among Isoniazid-Resistant Isolates

Genes	Total (225)	Wild-Type Probe Loss (R)	Frequency (%)	Mutant Probe Detected	Frequency (%)
inhA	91	WT1, WT2	32 (35.16)	MUT1, MUT2, MUT3A, MUT3B	59 (64.84)

katG	134	WT1	69 (51.49%)	MUT1, MUT2	65 (48.50)

WT = Wild-type probe; MUT = Mutant probe; R = Absence of hybridization to the corresponding wild-type probe (wild-type probe loss), indicating a mutation within the targeted region detected by the line probe assay.

Among the 91 *inhA*-positive samples, resistance was found in 64.84% cases due to the presence of a mutation, whereas in 35.16% cases resistance was due to the absence of the wild-type gene. On the other side, among the 134 *katG*-positive samples, 65 (48.50%) carried mutant-type alleles, while 69 (51.49%) displayed the wild-type pattern (WT1) (Figure 5).

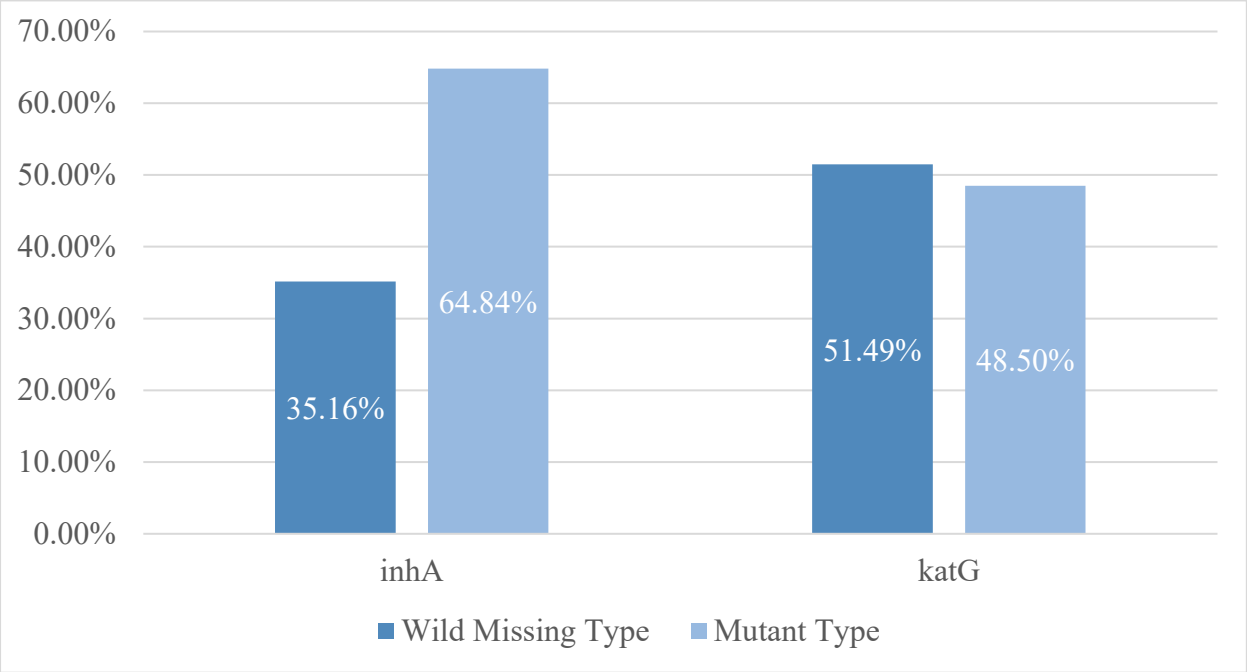

Figure 5: Distribution of wild-type loss and mutant-type patterns in the inhA and katG genes among isoniazid-resistant isolates

When combined mutation patterns were considered, S531L emerged as the most frequent individual rpoB mutation, occurring in 50 (51.02%) of the 98 mutant isolates. D516V was present in 30 (30.61%) isolates, followed by H526Y in 21 (21.43%) and H526D in 14 (14.29%) isolates. The cumulative frequencies exceeded 100% because several isolates harbored more than one mutation simultaneously (Table 5).

Table 5: Overall Frequency of Individual rpoB Mutations among Rifampicin-Resistant Isolates

Mutation	Frequency (n)	Percentage (%)
D516V	30	30.61
H526Y	21	21.43
H526D	14	14.29
S531L	50	51.02

Table 6 lists the specific amino acid substitutions identified in the studied isolates, all of which were located in the same protein chain (Chain C). Histidine (HIS) at position 445 was replaced by either Tryptophan (TYP) or Aspartate (ASP), while Aspartate (ASP) at position 435 was replaced by Valine (VAL), and Serine (SER) at position 450 was replaced by Leucine (LEU). These substitutions represent potential markers of isoniazid resistance, particularly in the *katG* gene, where mutations at positions 435 and 445 are well-documented to reduce catalase-peroxidase activity (Table 6).

Table 6: Amino Acid Substitutions Selected for Protein Structural Analysis

Wild-Type Residue	Codon Position	Mutant Residue	Protein Chain	Corresponding LPA Mutation
ASP	435	VAL	C	D516V
HIS	445	TRP	C	H526Y
HIS	445	ASP	C	H526D

SER	450	LEU	C	S531L

HIS=Histidine **ASP**=Aspartate **SER**=Serine **TRP**=Tryptophan **VAL**=Valine **LEU**=Leucine

Note: Amino acid substitutions were selected from the predominant resistance-associated mutations identified by line probe assay and subsequently subjected to structural modelling to evaluate their potential effects on protein stability and dynamics.
A very high resistance level was observed with the rpoMUT3 mutation, which affects the S531L amino acid, and this pattern was more frequent compared to others, whereas rpoMUT1 affects the D516V amino acid and occurs at a moderate level of resistance (Table 7).

Table 7: Effect of Mutation bands on Amino Acid change and Resistance level

MUT Band	Codon	Amino Acid Change	Resistance Level
rpoMUT1	516	D516V	Moderate
rpoMUT2a	526	H526Y	High
rpoMUT2b	526	H526D	High
rpoMUT3	531	S531L	Very High (most common)

Predicted structural effects of resistance-associated mutations on protein stability showed that all identified mutations reduced protein stability to varying degrees (Table 8). The H445D mutation exhibited the greatest destabilizing effect (ΔΔG = −1.9 kcal/mol), followed by H445Y (ΔΔG = −1.4 kcal/mol), S450L (ΔΔG = −1.1 kcal/mol), D435V (ΔΔG = −0.8 kcal/mol), and S315T (ΔΔG = −0.7 kcal/mol), which was only mildly destabilizing. Structural deviation, as measured by RMSD, was highest for H445D (3.2 Å), followed by H445Y (2.8 Å), S450L (2.6 Å), D435V (2.3 Å), and S315T (1.9 Å). RMSF analysis demonstrated high flexibility for H445D and H445Y, moderate flexibility for D435V and S450L, and low flexibility for S315T. Similarly, hydrogen bond analysis indicated a marked reduction in hydrogen bonding for H445D and H445Y, a moderate reduction for D435V and S450L, and only minimal changes for S315T. Overall, H445D and H445Y were predicted to produce the most pronounced structural disruption and instability, whereas S315T largely preserved protein folding despite mild destabilization

Table 8: Predicted structural effects of identified resistance mutations on protein stability

Mutation	ΔΔG (kcal/mol)	Stability	RMSD	RMSF peak	H-bond change	Interpretation
D435V	−0.8	destabilizing	2.3	moderate	↓	moderate instability
H445Y	−1.4	destabilizing	2.8	high	↓↓	conformational disruption
H445D	−1.9	destabilizing	3.2	high	↓↓	marked instability
S450L	−1.1	destabilizing	2.6	moderate	↓	altered drug-binding pocket
S315T	−0.7	mildly destabilizing	1.9	low	minimal	preserved folding

DISCUSSION

This study presents a comprehensive assessment of rifampicin- and isoniazid-resistance-associated mutation patterns among 266 pediatric patients with multidrug-resistant tuberculosis in Khyber Pakhtunkhwa, Pakistan. Rifampicin resistance was detected by line probe assay (LPA) in 211 (79.32%) isolates, while isoniazid resistance was identified in 225 (84.58%) isolates. The results indicate substantial heterogeneity in resistance-associated mutation patterns, particularly within the rpoB gene, with both individual and combined mutations observed. These findings are significant because molecular data on drug-resistant tuberculosis in children are limited, especially in Pakistan, and the distribution of resistance-associated mutations may differ by geographical region, circulating strains, treatment history, and selective pressure.
The proportion of rifampicin-resistant isolates detected by line probe assay (LPA) in this study (79.32%) exceeded that identified by phenotypic drug susceptibility testing (DST) (72.9%). Similarly, isoniazid resistance was detected in 84.58% of isolates by LPA, compared to 81.9% by DST. These findings underscore the utility of molecular testing for the rapid identification of resistance-associated genetic changes. The GenoType MTBDRplus assay targets specific regions of *rpoB*, *katG*, and *inhA, providing* resistance information more rapidly than conventional culture-based DST. Previous research has demonstrated strong concordance between LPA and phenotypic DST, although discrepancies may arise because genotypic and phenotypic methods evaluate different aspects of resistance. For example, a 2021 pediatric study found that GenoType MTBDRplus identified diverse resistance patterns and revealed that certain rifampicin-resistance-associated mutations could be missed by Xpert

MTB/RIF, as these mutations occurred outside its primary target regions.[14] Another study comparing MTBDRplus with phenotypic DST reported high accuracy for detecting rifampicin and isoniazid resistance, while also noting discordant results that warrant further investigation.[15] Additionally, a 2023 systematic review and meta-analysis concluded that molecular and phenotypic approaches yield broadly comparable estimates of resistance-associated mutations, but highlighted substantial geographical variation in mutation frequencies.[16]

A key finding in this study was that 113 (53.55%) of the 211 rifampicin-resistant isolates lacked one or more wild-type rpoB probes without a corresponding detectable mutant probe. These patterns suggest resistance-associated variation within the targeted *rpoB* region, although the available mutant probes cannot identify the specific mutation. This outcome indicates a limitation of line probe assay (LPA) technology, rather than evidence that these isolates are genetically wild-type. Other studies have documented similar unresolved patterns. For example, in a Georgian multidrug-resistant tuberculosis (MDR-TB) cohort, researchers could not identify the specific mutation responsible for rifampicin resistance in 17.4% of isolates because no corresponding mutant probe was detected.[17] A recent study from Brazil also demonstrated that a proportion of rifampicin-resistant isolates had inferred *rpoB* mutations that required sequencing for definitive characterization.[18] Furthermore, studies comparing LPA with sequencing have shown that uncommon mutations may remain undetected or unresolved by standard MTBDRplus mutation probes.[19] The relatively high proportion of unresolved *rpoB* patterns in this cohort may therefore reflect mutations not specifically represented by the assay. This finding underscores the need for future sequencing-based studies in KP to characterize these unresolved resistance-associated patterns.

Among the 98 isolates with identifiable *rpoB* mutant patterns, S531L was the most prevalent individual mutation, detected in 37 (37.75%) isolates. D516V was identified in 19 (19.39%) isolates, and H526D in 7 (7.14%) isolates. Considering combined mutations, S531L was present in 50 (51.02%) isolates, D516V in 30 (30.61%), H526Y in 21 (21.43%), and H526D in 14 (14.29%). The predominance of S531L aligns with established molecular epidemiology of rifampicin resistance. A 2023 systematic review and meta-analysis identified S531L as the most prevalent *rpoB* resistance-associated mutation globally, with a pooled frequency of approximately 13.5%.[16] A 2023 study of clinical *M. tuberculosis* isolates using line probe assay (LPA) also identified S531L as one of the predominant rifampicin-resistance-associated mutations.[20] Similarly, a study evaluating multidrug-resistant tuberculosis (MDR-TB) isolates using GenoType MTBDRplus reported S531L as the most frequent *rpoB* mutation.[21] The consistent predominance of S531L across diverse populations may be attributed to the biological fitness of strains harboring this mutation and its capacity to confer substantial rifampicin resistance while preserving sufficient bacterial fitness for persistence and transmission.

The detection of D516V, H526Y, and H526D mutations in this cohort further indicates that rifampicin resistance is genetically heterogeneous. Although these mutations occur less frequently than S531L, each constitutes a recognized resistance-associated alteration within the *rpoB* rifampicin resistance-determining region. A large molecular study from the southern coastal region of India identified S531L as the predominant mutation, followed by H526Y, D516V, and H526D.[22] Similarly, a study from Côte d'Ivoire utilizing MTBDRplus detected D516V, H526Y, H526D, and S531L among rifampicin-resistant isolates, although the relative distribution differed from the present findings.[23] Another investigation of multidrug-resistant tuberculosis (MDR-TB) using MTBDRplus also demonstrated that mutations at codons 531, 516, and 526 represent the principal rifampicin-resistance patterns.[24] Variations in the relative frequency of these mutations across studies may reflect differences in circulating *Mycobacterium tuberculosis* lineages, transmission dynamics, prior treatment exposure, and local antimicrobial pressures. Consequently, consider regional mutation profiles rather than assuming a single global pattern applies uniformly across populations.

Identifying combined *rpoB* mutations is a significant finding. The D516V + S531L combination was found in 7 (7.14%) isolates, while H526D + S531L was observed in 3 (3.06%) isolates. No isolate exhibited three simultaneous *rpoB* mutations. Multiple resistance-associated mutations have been documented in molecular studies of multidrug-resistant tuberculosis (MDR-TB), although their prevalence varies across populations. Studies utilizing MTBDRplus have reported combinations involving various *rpoB* mutation sites, and sequencing studies have shown that some complex line probe assay (LPA) patterns may indicate additional or uncommon mutations not detected by the assay.[19,25] The presence of combined mutations in this cohort may reflect the sequential accumulation of resistance-associated changes or the existence of genetically heterogeneous bacterial populations. However, since LPA does not provide nucleotide-level sequencing data, it cannot be determined whether all detected mutation patterns occur within the same bacterial genome or represent mixed populations. Consequently, interpret these results as combined LPA mutation patterns, not definitive evidence of multiple mutations within a single bacterial genome.

The distribution of isoniazid resistance exhibited distinct molecular patterns. Among 225 isolates identified as isoniazid resistant by line probe assay (LPA), 91 displayed *inhA*-associated patterns and 134 exhibited *katG*-associated patterns. In the *inhA* group, 59 isolates (64.84%) showed identifiable mutant patterns, while 32 (35.16%) lacked a corresponding detectable mutant pattern. For *katG*-associated isolates, 65 (48.50%) demonstrated identifiable mutant patterns, whereas 69 (51.49%) presented unresolved wild-type probe patterns. These results suggest that *katG*-associated resistance constitutes a significant component of isoniazid resistance in this pediatric multidrug-resistant tuberculosis (MDR-TB) cohort. Furthermore, a substantial proportion of resistance-associated patterns could not be attributed to specific mutations using LPA.

The predominance of *katG*-associated resistance observed in this study is biologically plausible and aligns with previous molecular research. A 2023 systematic review and meta-analysis identified katG S315T as the most prevalent isoniazid-resistance-associated mutation globally, followed by the *inhA* promoter C-15T mutation.[16] Furthermore, molecular data from Khyber Pakhtunkhwa provide strong support for these findings. Khan et al., through whole-genome sequencing of 51 phenotypically isoniazid-resistant isolates from KP, reported *katG* S315T in 37 isolates (72.5%), while the *inhA* promoter -15C>T mutation was detected in 13 isolates (25.5%).[10] Therefore, the predominance of *katG*-associated resistance in the pediatric cohort is consistent with the broader molecular epidemiology of isoniazid resistance in KP.

The high frequency of *katG*-associated resistance is clinically significant, as mutations in *katG*, particularly S315T, are typically linked to high-level isoniazid resistance. In contrast, mutations in the *inhA* promoter are generally associated with lower-level isoniazid resistance and may contribute to cross-resistance with thioamides. Research on resistance-associated mutations in *M. tuberculosis* has shown that *katG* and *inhA* alterations constitute primary mechanisms of isoniazid resistance, although sequencing has revealed additional mutations outside the regions targeted by standard line probe assays (LPA).[26] This finding may account for the substantial number of isoniazid-resistant isolates in the present study that lacked a specific mutant probe pattern.

The significant role of *inhA*-associated resistance in this cohort warrants attention. Of the 91 isolates exhibiting *inhA*-associated resistance patterns, 59 (64.84%) displayed detectable mutant patterns. The *inhA* promoter represents a critical resistance mechanism, as mutations in its regulatory region can elevate InhA target expression and diminish susceptibility to isoniazid. A 2023 meta-analysis identified the C-15T mutation in the *inhA* promoter as the second most prevalent isoniazid-resistance-associated mutation globally.[16] Similarly, the KP whole-genome sequencing study reported *inhA* promoter -15C>T as a key local resistance-associated mutation, present in 13 of 51 isolates.[10] Another molecular study utilizing MTBDRplus found *inhA* C-15T among the primary resistance-associated patterns in MDR-TB isolates.[25] The detection of both *katG*- and *inhA*-associated patterns in this cohort underscores the necessity of examining both targets when investigating pediatric MDR-TB.

A notable finding was the relatively high proportion of unresolved *katG* and *inhA* patterns. Within the *katG*-associated group, 69 cases (51.49%) lacked a corresponding detectable mutant probe, while 32 cases (35.16%) in the *inhA*-associated group exhibited no identifiable mutant pattern. This result should not be construed as evidence for the absence of genetic resistance. Instead, it highlights the limitations inherent in targeted molecular assays. The GenoType MTBDRplus assay examines selected regions and common mutations, yet resistance can arise from alterations outside these predefined targets. Sequencing studies have confirmed this limitation. For example, the KP whole-genome sequencing study identified several additional *katG* mutations and other candidate resistance-associated alterations beyond the common LPA targets.[10] Similarly, sequencing-based evaluations of MTBDRplus have shown that rare *katG* mutations may not be detected by the assay.[19] Consequently, the unresolved patterns observed in this study underscore the need for future sequencing-based investigations, rather than serving as evidence against a genetic basis for resistance.

The coexistence of resistance-associated mutations across multiple genes is pertinent to interpreting multidrug-resistant tuberculosis (MDR-TB) in this cohort. As MDR-TB is defined by resistance to at least rifampicin and isoniazid, combinations of *rpoB* and *katG/inhA* resistance patterns are anticipated. Nevertheless, the specific mutation combinations can differ substantially across isolates. A study of treatment-naïve and recurrent MDR-TB cases identified differences in *rpoB*, *katG*, and inhA patterns by treatment history, with S531L as the most prevalent rpoB mutation and katG mutations significantly contributing to isoniazid resistance.[25] These findings indicate that resistance patterns may result from both transmitted resistance and resistance acquired during prior treatment. Since this study focused on pediatric MDR-TB and was not designed to differentiate between transmitted and acquired resistance, these mechanisms cannot be determined from the current line probe assay (LPA) data alone.

The inclusion of a pediatric population constitutes a notable strength of this study. The majority of participants were adolescents aged 10–14 years, and the cohort comprised a relatively large number of children with multidrug-resistant tuberculosis (MDR-TB). Molecular resistance data in pediatric populations remain less abundant than in adults due to challenges in obtaining microbiological confirmation in children. A 2021 pediatric study utilizing GenoType MTBDRplus demonstrated that molecular testing can identify significant resistance patterns in children and reported S531L-associated *rpoB* resistance as the predominant rifampicin-resistance pattern.[14] The observed similarity in the predominance of S531L between that pediatric cohort and the present study indicates that this mutation may be particularly significant in pediatric MDR-TB populations. Nevertheless, the substantially higher proportion of unresolved *rpoB* patterns in this cohort underscores the need for ongoing local molecular surveillance and additional sequencing in KP.

Strengths: The present study demonstrates several strengths. It specifically addresses pediatric multidrug-resistant tuberculosis (MDR-TB), incorporates a relatively large cohort from Khyber Pakhtunkhwa, and offers detailed characterization of individual and combined resistance-associated patterns in *rpoB*, *katG*, and *inhA*. Furthermore, the inclusion of both phenotypic drug susceptibility testing (DST) and line probe assay (LPA) results enables a direct comparison between conventional and molecular diagnostic approaches.

Limitations: Several limitations are present in this study. First, line probe assay (LPA) detects only predefined mutations and does not provide a comprehensive characterization of the genetic basis of resistance. Second, the specific mutations underlying unresolved wild-type probe patterns could not be identified due to the absence of

sequencing. Third, LPA cannot reliably distinguish whether combined mutation patterns reflect multiple mutations within a single organism or the presence of mixed or heteroresistant populations. Finally, the study population comprised children receiving care through multidrug-resistant tuberculosis (MDR-TB) services in Khyber Pakhtunkhwa (KP), which may limit the generalizability of the findings to all pediatric tuberculosis patients in Pakistan.

This study reveals significant diversity in rifampicin- and isoniazid-resistance-associated mutation patterns among pediatric multidrug-resistant tuberculosis (MDR-TB) patients in Khyber Pakhtunkhwa. The S531L mutation was the most prevalent rpoB mutation identified, while katG-associated mutations constituted a major component of isoniazid resistance. The detection of D516V, H526Y, H526D, and combined *rpoB* mutation patterns indicates substantial molecular heterogeneity. Furthermore, a considerable proportion of isolates exhibited loss of *rpoB* wild-type probes without a corresponding detectable mutant probe, underscoring an unresolved aspect of rifampicin resistance that cannot be fully characterized by line probe assay (LPA) alone. These results support the ongoing use of rapid molecular diagnostics for pediatric MDR-TB and underscore the necessity for sequencing-based studies to identify unresolved and rare resistance-associated mutations in this region. Developing region-specific mutation profiles may strengthen molecular surveillance, support rapid diagnostics, and inform more effective strategies to control pediatric MDR-TB in Pakistan.

CONCLUSION

This study reveals significant molecular heterogeneity in rifampicin- and isoniazid-resistance-associated patterns among pediatric patients with multidrug-resistant tuberculosis (MDR-TB) in Khyber Pakhtunkhwa, Pakistan. Rifampicin resistance was primarily associated with identifiable *rpoB* mutations, with S531L being the most prevalent, followed by D516V and H526D. Combined *rpoB* mutation patterns were also detected. For isoniazid resistance, katG-associated mutations constituted a major component, along with *inhA*-associated resistance patterns. Notably, a substantial proportion of rifampicin-resistant isolates exhibited loss of one or more *rpoB* wild-type probes without a corresponding detectable mutant probe, suggesting resistance-associated changes not specifically characterized by line probe assay (LPA). These results underscore the diversity of resistance-associated patterns in pediatric MDR-TB and the critical role of rapid molecular testing for their identification. The high proportion of unresolved mutation patterns further indicates the necessity for sequencing-based investigations to detect less common or uncharacterized resistance-associated mutations in this population. Collectively, these findings provide valuable regional molecular data that may enhance drug-resistance surveillance, interpretation of molecular diagnostic results, and understanding of pediatric MDR-TB in Khyber Pakhtunkhwa.

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