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PREVALENCE AND MOLECULAR CHARACTERIZATION OF DRUG-RESISTANT MYCOBACTERIUM TUBERCULOSIS USING GENEXPERT MTB/RIF ULTRA AND MTB/XDR ASSAYS ACROSS AZAD JAMMU AND KASHMIR, PAKISTAN (2025)

Laiba Tahir[1] , Dr. Tahseen Ismail[2] , Abdullah Sajjad[3] , Iman Fatima[4] , Dr. Abeer Vaqar[5] , Muhammad Nauman Sharif*[6](Corresponding author) , Noor-ul-iza[7] , Sabah mansoor[8]

[1]Department of Microbiology, Mirpur University of Science and Technology (MUST) laibatahirpk@gmail.com
[2]Coordinator, Department of Microbiology, Mirpur University of Science and Technology (MUST) tahseen.micro@must.edu.pk
[3]MBBS, Bahria University of Health Sciences Campus Karachi (BUHSCK) abdsajjad1234@gmail.com
[4]Department of Microbiology, Mirpur University of Science and Technology (MUST) imanfatimaa255@gmail.com
[5]MBBS, FCPS (Peads), Assistant Professor (Pediatrics), BUMC/KRL Hospital Islamabad abeerhaq@hotmail.com
[6]State TB Reference Laboratory, Div HQs Teaching Hospital Mirpur AJK. nauman.sharif5632102@gmail.com
[7]Department of Microbiology, Mirpur University of Science and Technology (MUST) noorizayy@gmail.com
[8]Assistant Professor, Department of allied sciences, University of Home Economics Lahore. sabahgillani.as@uhe.edu.pk

Abstract:

Background: Tuberculosis (TB) remains a critical public health challenge in Pakistan, yet comprehensive molecular epidemiological data from Azad Jammu and Kashmir (AJK) are lacking. This study aimed to determine the prevalence and molecular drug resistance profiles of Mycobacterium tuberculosis across AJK using the GeneXpert MTB/RIF Ultra and MTB/XDR assays during 2025**. Methods:** A retrospective, cross-sectional study was conducted across 14 National TB Control Programme (NTP)-designated facilities spanning 10 districts in AJK from January to December 2025. A diagnostic cascade was utilized: MTB/RIF Ultra was performed on 24,988 presumptive pulmonary TB patients, followed by MTB/XDR testing on a subset of 50 cases (18 rifampicin-resistant [RR] and 32 rifampicin-sensitive) to characterize extended resistance. **Results:** The overall MTB positivity rate was 11.1% (n=2,771), with Trace-level semiquantitative results constituting 17.8% of positives. Rifampicin resistance was identified in 21 cases (0.93% rate). Alarmingly, 61.9% of RR-TB cases were new patients, indicating active primary transmission of resistant strains. Spatial analysis revealed significant heterogeneity: Jhelum Valley recorded the highest district-level positivity rate (16.9%), while Mirpur disproportionately accounted for 47.6% of all RR-TB cases. MTB/XDR analysis exposed high rates of extended resistance; among RR-TB cases, 50.0% exhibited isoniazid (INH) resistance (MDR-TB) and 35.7% showed fluoroquinolone (FQ) resistance (pre-XDR-TB). Crucially, among RIF-sensitive cases, INH mono-resistance and FQ resistance were detected in 19.2% and 27.3% of interpretable results, respectively. **Conclusion:** AJK faces a substantial and geographically heterogeneous TB burden, marked by significant primary RR-TB transmission and hidden INH and FQ resistance among routinely classified drug-sensitive patients. Expanding reflex MTB/XDR testing, intensifying contact tracing, and strengthening remote specimen transportation networks are urgently needed to optimize targeted TB control strategies in the region.

Keywords: Mycobacterium tuberculosis, GeneXpert MTB/RIF Ultra, Xpert MTB/XDR, Multidrug-resistant TB, Pre-XDR-TB, Molecular epidemiology, Azad Jammu and Kashmir, Pakistan.

Introduction:

Tuberculosis (TB), caused by the acid-fast bacillus Mycobacterium tuberculosis, remains one of the top ten leading causes of mortality worldwide [1]. According to the World Health Organization (WHO) Global Tuberculosis Report 2024, an estimated 10.7 million people fell ill with TB globally, resulting in 1.23 million deaths. This staggering burden marks TB's resurgence as the leading infectious disease killer, surpassing COVID-19 [1].

The emergence of drug-resistant M. tuberculosis is primarily driven by mutations in key resistance-conferring genes: rpoB (rifampicin), katG and inhA (isoniazid), embB (ethambutol), pncA (pyrazinamide), and rrs and eis (aminoglycosides) [2]. These genetic alterations arise either de novo through inadequate or interrupted treatment (acquired resistance) or via direct transmission from an index case (primary resistance), each carrying distinct epidemiological implications for TB control programs [3]. The co-occurrence of rpoB and katG/inhA mutations

defines the multidrug-resistant (MDR-TB) phenotype. Furthermore, the acquisition of fluoroquinolone and aminoglycoside resistance characterizes pre-extensively and extensively drug-resistant TB (pre-XDR and XDR-TB), representing progressive therapeutic crises associated with significantly poorer clinical outcomes [4].

Historically, conventional diagnostic methods such as smear microscopy and mycobacterial culture have been central to TB detection. However, these approaches possess critical limitations. Smear microscopy exhibits low sensitivity [5], particularly in paucibacillary cases, including children, individuals with HIV co-infection, and those with extrapulmonary TB. While culture remains the diagnostic gold standard [6], its slow turnaround time—often requiring several weeks due to the slow growth rate of M. tuberculosis—delays critical clinical decisions. These limitations are particularly consequential in resource-limited settings like Azad Jammu and Kashmir (AJK), where constrained diagnostic infrastructure and delayed confirmation of drug-resistant TB exacerbate community transmission, treatment failure, and overall disease burden [7].

To overcome these diagnostic bottlenecks, the WHO has endorsed rapid molecular tools such as the GeneXpert MTB/RIF Ultra. This cartridge-based nucleic acid amplification test (NAAT) utilizes real-time PCR within a closed, automated system to simultaneously detect M. tuberculosis complex DNA and rifampicin resistance within 80–90 minutes. By targeting the rpoB gene alongside multicopy insertion sequences (IS6110 and IS1081), the Ultra assay significantly enhances sensitivity, particularly for paucibacillary and vulnerable patient populations [8]. Building on this technological foundation, recent advancements have introduced the Xpert MTB/XDR assay, which enables the rapid detection of resistance to second-line anti-tubercular drugs. This assay simultaneously identifies mutations associated with resistance to isoniazid, fluoroquinolones, ethionamide, and injectable agents (amikacin, kanamycin, and capreomycin) by targeting genes such as katG, inhA promoter, gyrA, gyrB, rrs, and eis promoter [9]. Compared to conventional phenotypic drug susceptibility testing, the MTB/XDR assay drastically reduces turnaround times, facilitating the prompt initiation of appropriate treatment regimens in high-burden, resource-limited settings [10].

Pakistan bears a disproportionate share of the global TB burden, ranking fifth among the 30 high-burden countries. The nation sees approximately 510,000 new TB cases and 15,000 drug-resistant cases annually, accounting for 61% of the TB burden in the WHO Eastern Mediterranean Region and harboring the fourth-highest MDR-TB prevalence globally. Within this national context, AJK, with a population of approximately 4.5 million, presents a distinct and highly vulnerable epidemiological landscape. The region's cold mountainous climate [11], limited healthcare infrastructure, restricted access to molecular diagnostics, population displacement, and cross-border movement with adjacent high-burden areas [12] collectively drive the escalating TB burden.

Despite this vulnerability, existing literature on the TB burden in AJK is largely restricted to isolated regions such as Muzaffarabad and Mirpur [11,13]. Comprehensive, region-wide epidemiological data detailing M. tuberculosis prevalence and extended drug resistance profiles across AJK for the year 2025 remain absent. This critical knowledge gap severely hinders evidence-based planning and resource allocation for AJK's TB control programs. Therefore, the present study aimed to analyze the epidemiological landscape of TB in AJK during 2025 by deploying a dual-assay molecular diagnostic cascade. By utilizing the Xpert MTB/RIF Ultra and Xpert MTB/XDR assays, this study sought to assess the prevalence of M. tuberculosis and characterize molecular markers associated with both first- and second-line drug resistance, thereby generating vital, comprehensive molecular epidemiological data for this underrepresented region.

Literature Review:

Tuberculosis (TB) remains a formidable global public health challenge, consistently ranking among the top ten leading causes of death worldwide. According to the World Health Organization (WHO), an estimated 10.7 million people fell ill with TB globally, resulting in 1.23 million deaths, reaffirming its status as the leading infectious disease killer [14]. The emergence of drug-resistant Mycobacterium tuberculosis is primarily driven by genetic mutations in key resistance-conferring genes, such as rpoB for rifampicin, and katG and inhA for isoniazid [15], [16]. The co-occurrence of these mutations defines multidrug-resistant TB (MDR-TB), while the additional acquisition of resistance to fluoroquinolones and second-line injectable agents characterizes pre-extensively drug-resistant (pre-XDR) and extensively drug-resistant (XDR) TB, representing progressive therapeutic crises with significantly worse clinical outcomes [17].

Conventional diagnostic methods, namely smear microscopy and mycobacterial culture, have historically formed the backbone of TB detection. However, these approaches possess critical limitations. Smear microscopy exhibits notably low sensitivity, particularly in paucibacillary cases, pediatric populations, and patients co-infected with HIV [18]. Although mycobacterial culture is considered the diagnostic gold standard, it is highly time-consuming due to the slow growth rate of M. tuberculosis, often requiring several weeks to yield actionable results [19]. To address these diagnostic delays, the WHO has endorsed rapid molecular diagnostic tools, most notably the GeneXpert MTB/RIF Ultra assay. This cartridge-based nucleic acid amplification test (NAAT) detects M. tuberculosis complex DNA and rifampicin resistance within 80–90 minutes by targeting the rpoB gene and multicopy insertion sequences (IS6110 and IS1081), thereby markedly improving sensitivity in paucibacillary and vulnerable patient populations [20].

Recent advancements in molecular diagnostics have further expanded the capacity to detect resistance to second-line anti-tubercular drugs rapidly [21]. The Xpert MTB/XDR assay represents a critical evolution in this domain, simultaneously detecting resistance-associated mutations linked to isoniazid, fluoroquinolones, ethionamide, and second-line injectable agents (e.g., amikacin, kanamycin, and capreomycin) [22]. By targeting specific mutations in genes such as katG, inhA promoter, gyrA, gyrB, rrs, and eis promoter regions, the MTB/XDR assay provides rapid molecular characterization of pre-XDR and XDR-TB directly from clinical specimens. Compared to conventional phenotypic drug susceptibility testing, which can take weeks, the MTB/XDR assay offers a drastically reduced turnaround time, facilitating the earlier initiation of appropriate, life-saving treatment regimens, particularly in high-burden and resource-limited settings [23].

Pakistan bears a disproportionate share of the global TB burden, ranking fifth among the 30 high-burden TB countries, with an estimated 510,000 new cases and 15,000 drug-resistant TB cases emerging annually [1]. Within this national context, Azad Jammu and Kashmir (AJK) presents a distinct and vulnerable epidemiological landscape. The region's cold mountainous climate, limited healthcare infrastructure, restricted access to molecular diagnostic facilities, and population displacement with cross-border movement have been identified as significant contributing factors to the increasing TB burden [14], [24]. While limited studies have examined the TB burden in specific divisions of AJK, such as Muzaffarabad and Mirpur, these investigations have been geographically constrained and have not utilized a comprehensive two-assay molecular diagnostic cascade [10], [25].

Consequently, a critical gap exists in the published literature regarding comprehensive, region-wide epidemiological data on M. tuberculosis prevalence and extended drug resistance profiles across all districts of AJK. The absence of such data severely hinders evidence-based planning and resource allocation for the National TB Control Programme (NTP) in the region [26]. Therefore, leveraging the combined diagnostic power of the GeneXpert MTB/RIF Ultra and MTB/XDR assays is essential to establish a robust molecular epidemiological baseline. This approach will not only characterize the prevalence of first- and second-line drug resistance but also inform targeted public health interventions, such as enhanced contact tracing and optimized specimen transportation networks, to mitigate the transmission of drug-resistant TB in this underrepresented region

Methods

Study design

This was a retrospective, cross-sectional, descriptive-analytical study conducted to determine the prevalence of Mycobacterium tuberculosis and characterize drug resistance profiles among presumptive TB patients across Azad Jammu and Kashmir (AJK), Pakistan, during 2025. A two-assay molecular diagnostic cascade was employed: GeneXpert MTB/RIF Ultra for simultaneous M. tuberculosis detection and rifampicin resistance screening among all presumptive TB patients, followed by GeneXpert MTB/XDR for comprehensive characterization of isoniazid and fluoroquinolone resistance among selected cases

Study setting

The study was conducted across 14 NTP-designated GeneXpert diagnostic facilities in AJK — 12 public sector facilities operating under the National TB Control Programme (NTP) Pakistan and 2 private sector laboratories contracted under the Managed Care (MC) program. The facilities were distributed across eight districts of AJK: Mirpur, Bhimber, Kotli, Sudhnuti (Palandri), Poonch (Rawalakot), Bagh, Muzaffarabad, Haveli, Jhelum Valley (Hattian Bala), and Neelum Valley. GeneXpert testing was performed using GeneXpert Dx Systems equipped with both 4-module and 10-module instruments (Cepheid, Sunnyvale, CA, USA). Additionally, a Specimen Transportation Program (STP) facilitated sample collection from 32 remote health facilities across 5 districts that lacked on-site GeneXpert capacity, transporting specimens to designated testing laboratories.

Study period

Retrospective data were retrieved for the period January 2025 to December 2025, covering four complete quarters (Q1: January–March, Q2: April–June, Q3: July–September, Q4: October–December).

Study Population and Case Definitions

The study population comprised all presumptive pulmonary TB patients registered and tested at participating NTP GeneXpert facilities in AJK during 2025. Presumptive TB was defined per WHO criteria as any patient presenting with cough of two or more weeks with or without fever, night sweats, hemoptysis, or unexplained weight loss.(27)

The following case definitions were applied:

Category	Definition
MTB detected	GeneXpert MTB/RIF Ultra: MTB Detected result
Trace positive	MTB detected with Trace semiquantitative bacterial load
RIF sensitive (DS-TB)	MTB Detected, RIF Resistance NOT Detected

RIF resistant (RR-TB)	MTB Detected, RIF Resistance DETECTED
INH resistant	GeneXpert MTB/XDR: INH resistance detected
FQ resistant	GeneXpert MTB/XDR: Fluoroquinolone resistance detected
MDR-TB	RIF resistant + INH resistant
Pre-XDR-TB	MDR-TB + FQ resistant
New case	No prior anti-TB treatment history
Previously treated	One month or more prior anti-TB treatment

Diagnostic Procedures

GeneXpert MTB/RIF Ultra
All presumptive TB patients provided spot or early morning sputum specimens processed according to WHO standard operating procedures. Specimens were treated with GeneXpert sample reagent in a 2:1 ratio, vortexed for 15 seconds, incubated at room temperature for 15 minutes, and 2 ml of the processed sample was loaded into the GeneXpert Ultra cartridge. The instrument automatically performed integrated DNA extraction, real-time PCR amplification, and result interpretation within approximately 80 minutes. The assay simultaneously targeted IS6110 and IS1081 multicopy sequences for M. tuberculosis complex detection and five molecular beacon probes spanning the 81-bp rifampicin resistance-determining region (RRDR) of the rpoB gene for resistance detection. Semiquantitative bacterial load was reported as Trace, Low, Medium, or High based on cycle threshold (Ct) values.

GeneXpert MTB/XDR
Selected cases were further tested using the GeneXpert MTB/XDR assay (Cepheid, Sunnyvale, CA, USA) for comprehensive drug resistance characterization. The MTB/XDR assay detects resistance to isoniazid (INH) via mutations in katG and inhA genes, fluoroquinolones (FQ) via mutations in gyrA and gyrB genes, and aminoglycosides via mutations in rrs and eis genes. In this study, XDR testing was performed on two categories of cases: (a) confirmed RIF-resistant cases (n=18) for MDR-TB and pre-XDR-TB classification, and (b) a subset of RIF-sensitive cases (n=32) to detect isoniazid mono-resistance and fluoroquinolone resistance in drug-sensitive TB patients.

Data Collection
Data were retrospectively extracted from NTP GeneXpert laboratory registers and RIF-resistant case tracking registers maintained at participating facilities. For the MTB/RIF Ultra component, aggregate quarterly data were collected at facility level including total presumptive cases, total GeneXpert tests performed, MTB detected (including Trace), Trace results, RIF-interpretable results, and RIF resistance detected. For confirmed RR-TB cases, individual patient-level data were extracted from the dedicated RRD (Rifampicin Resistance Detected) case register including facility name, sample number, month of detection, patient age, sex, disease site, and history of previous TB treatment. For XDR testing, aggregate results were extracted for both RIF-resistant and RIF-sensitive subgroups including total tested, MTB detected, INH interpretable results, FQ interpretable results, INH resistant, and FQ resistant. Additionally, Specimen Transportation Program (STP) data were collected for AJK, capturing quarterly totals of transported samples, MTB detected, and RR detected from remote health facilities.

Statistical Analysis
Data were organized and analyzed using Microsoft Excel 2019 and IBM SPSS Statistics version 26.0. The overall prevalence of M. tuberculosis was calculated as the proportion of GeneXpert-positive cases among total presumptive TB patients tested, expressed as percentage with 95% confidence intervals calculated using the Wilson score method(15). RIF resistance rate was calculated as the proportion of RR-TB cases among all RIF-interpretable results. INH and FQ resistance rates were calculated as proportions of resistant cases among interpretable results within the XDR-tested subgroup separately for RIF-resistant and RIF-sensitive cases. Facility-wise and district-wise TB positivity and resistance rates were calculated and compared using Chi-square test. Quarterly trends in TB detection and resistance were analyzed across Q1–Q4 2025. Demographic characteristics of RIF-resistant cases — including age, sex, and treatment history — were described as frequencies and percentages. Fisher's exact test was used for comparisons where expected cell counts were below five. A p-value of less than 0.05 was considered statistically significant.

Results

Study Population and GeneXpert Testing Volume
During January 2025 to December 2025, a total of 24,988 presumptive pulmonary tuberculosis patients were tested across 14 NTP-designated GeneXpert MTB/RIF Ultra facilities in Azad Jammu and Kashmir — 12 public

sector and 2 private sector laboratories distributed across ten administrative districts. The distribution of testing volume and results across all facilities is presented in Table 1. The highest testing volume was recorded at CMH Rawalakot (n=3,935, 15.7% of total), followed by SRL AJK Mirpur (n=3,826, 15.3%) and AIMS Muzaffarabad (n=2,132, 8.5%). The lowest testing volume was recorded at DHQ Haveli (n=719, 2.9%). Public sector facilities accounted for 21,384 (85.6%) of total tests, while the two private sector laboratories contributed 2,604 (14.4%) tests.

Demographic and clinical characteristics

Of the 24,988 presumptive TB patients tested across Azad Jammu and Kashmir (AJK) in 2025, 2,771 (11.1%) were positive for M. tuberculosis. A proportion of 17.8% (492 cases) produced Trace-level semiquantitative results. For the 21 confirmed Rifampicin-Resistant TB (RR-TB) cases, individual patient-level data revealed a relatively even sex distribution, with males constituting 52.4% (n=11) and females 47.6% (n=10). The demographic burden was heaviest in the 50–59 years age group (38.1%, n=8), while the mean age overall was 47.6 ± 16.2 years, ranging from 16 to 76 years. All 21 RR-TB patients were diagnosed with pulmonary TB (PTB). Analysis of treatment history showed that 61.9% (n=13) were new cases indicating primary resistance, while 38.1% (n=8) had documented histories of prior anti-TB treatment.

Table 1: Demographic and Clinical Characteristics of Confirmed RR-TB Cases, AJK 2025 (n=21)

Characteristic	N	%
Sex		
Male	11	52.4%
Female	10	47.6%
Age group (years)		
<20	1	4.8%
20–29	2	9.5%
30–39	4	19.0%
40–49	3	14.3%
50–59	8	38.1%
≥60	3	14.3%
Mean age ± SD	47.6 ± 16.2 years	—
Age range	16–76 years	—
Disease site		
Pulmonary TB (PTB)	21	100%
Extrapulmonary TB	0	0%
Treatment history		
New cases — primary resistance	13	61.9%

Characteristic	N	%
Previously treated — acquired resistance	8	38.1%

Drug resistance profile

Rifampicin resistance interpretability was achieved for 2,258 (81.5%) of the MTB-positive cases. Among these, rifampicin resistance was detected in 21 cases, resulting in an RR-TB rate of 0.93%. Extended drug resistance characterization via the GeneXpert MTB/XDR assay on a subset of 50 cases (18 RIF-resistant, 32 RIF-sensitive) revealed significant extended resistance patterns. Among the interpretable RIF-resistant cases, Isoniazid (INH) resistance was found in 50.0% (indicating MDR-TB), and Fluoroquinolone (FQ) resistance in 35.7% (indicating pre-XDR-TB). Notably, among RIF-sensitive cases, INH mono-resistance was detected in 19.2%, and FQ resistance in 27.3%. Across all 50 XDR-tested samples, combined INH resistance was 30.0% and FQ resistance was 30.6%.

Table 2: GeneXpert MTB/XDR Results Stratified by Rifampicin Resistance Status, AJK 2025

Parameter	RIF-Resistant (n=18)	RIF-Sensitive (n=32)	Combined Total (n=50)
MTB detected n (%)	15 (83.3%)	25 (78.1%)	40 (80.0%)
INH interpretable n (%)	14 (77.8%)	26 (81.3%)	40 (80.0%)
INH resistant n (%)	7 (50.0%)	5 (19.2%)	12 (30.0%)
INH sensitive n (%)	7 (50.0%)	21 (80.8%)	28 (70.0%)
FQ interpretable n (%)	14 (77.8%)	22 (68.8%)	36 (72.0%)
FQ resistant n (%)	5 (35.7%)	6 (27.3%)	11 (30.6%)
FQ sensitive n (%)	9 (64.3%)	16 (72.7%)	25 (69.4%)
Resistance classification	MDR-TB / Pre-XDR-TB	INH mono-resistance / FQ resistance	

Spatial and temporal distribution

Significant geographic heterogeneity in M. tuberculosis prevalence was observed across the 10 AJK districts. Muzaffarabad contributed the highest absolute burden with 554 confirmed cases (20.0% of all positives), while Jhelum Valley recorded the highest district-level positivity rate at 16.9%. RR-TB cases were highly concentrated, with Mirpur district accounting for 47.6% (n=10) of all resistant cases, despite not having the highest overall testing volume. Conversely, five districts recorded zero RR-TB cases throughout the year.

Quarterly analysis indicated that testing volume peaked in Q3 (n=6,553), whereas the MTB positivity rate peaked in Q2 (12.3%). RR-TB cases were identified throughout all four quarters, with the highest concentration (33.3%, n=7) occurring in Q3. Additionally, the Specimen Transportation Program (STP) facilitated testing for 32 peripheral facilities, transporting 2,883 specimens (11.5% of total volume) and successfully identifying 256 MTB-positive and 4 RR-TB cases that might otherwise have lacked access to molecular diagnostic services.

Table 3: District-wise M. tuberculosis Prevalence and Rifampicin Resistance, AJK 2025

District	Total Tested	MTB Positive n (%)	95% CI	RIF Resistant n (%)
Muzaffarabad	5,472	554 (10.1%)	9.3–10.9%	6 (1.1%)

District	Total Tested	MTB Positive n (%)	95% CI	RIF Resistant n (%)
Mirpur	3,826	502 (13.1%)	12.1–14.2%	10 (2.0%)
Kotli	3,451	398 (11.5%)	10.5–12.6%	2 (0.5%)
Bagh	3,024	347 (11.5%)	10.3–12.7%	0 (0%)
Poonch	3,935	293 (7.4%)	6.6–8.3%	2 (0.7%)
Bhimber	1,620	225 (13.9%)	12.2–15.7%	0 (0%)
Jhelum Valley	968	164 (16.9%)	14.6–19.5%	1 (0.6%)
Sudhnuti	1,075	114 (10.6%)	8.8–12.6%	0 (0%)
Haveli	719	97 (13.5%)	11.1–16.3%	0 (0%)
Neelum	898	77 (8.6%)	6.9–10.6%	0 (0%)
Total	**24,988**	**2,771 (11.1%)**	**10.71–11.48%**	**21 (0.93%)**

Table 4: Quarterly Distribution of GeneXpert MTB/RIF Ultra Results, AJK 2025

Quarter	Period	Total Tested	MTB Detected n (%)	RR-TB n (% of annual)
Q1	Jan–Mar 2025	6,324	697 (11.0%)	5 (23.8%)
Q2	Apr–Jun 2025	6,106	752 (12.3%)	5 (23.8%)
Q3	Jul–Sep 2025	6,553	730 (11.1%)	7 (33.3%)
Q4	Oct–Dec 2025	6,003	592 (9.9%)	4 (19.0%)
Total	**Jan–Dec 2025**	**24,988**	**2,771 (11.1%)**	**21 (100%)**

DISCUSSION

Principal Findings

This retrospective, cross-sectional study provides the first comprehensive molecular epidemiological characterisation of tuberculosis across all ten districts of Azad Jammu and Kashmir using a two-assay GeneXpert cascade — GeneXpert MTB/RIF Ultra for simultaneous M. tuberculosis detection and rifampicin resistance screening, followed by GeneXpert MTB/XDR for extended drug resistance profiling — during the calendar year 2025. A total of 24,988 presumptive pulmonary TB patients were tested across 14 NTP-designated facilities, yielding an overall MTB positivity rate of 11.09% (95% CI: 10.71–11.48%), an RR-TB rate of 0.93% (95% CI: 0.61–1.42%), and critically, high rates of isoniazid and fluoroquinolone resistance among both rifampicin-resistant and rifampicin-sensitive cases subjected to XDR analysis. Collectively, these findings characterise AJK as a region with a substantial, geographically heterogeneous, and molecularly complex TB burden that demands urgently differentiated programmatic responses from the National TB Control Programme.

Overall TB Burden and Testing Coverage

The overall MTB positivity rate of 11.09% observed in this study exceeds the national programmatic GeneXpert positivity range of approximately 7–9% typically reported in Pakistani NTP data, suggesting that AJK carries a disproportionately higher TB burden relative to the national average.(1) This elevated rate likely reflects a

combination of genuine disease burden driven by AJK-specific risk factors — including the region's cold mountainous climate, limited healthcare infrastructure, restricted access to molecular diagnostics, population displacement, and cross-border movement with adjacent high-burden areas — and the success of active presumptive case-finding strategies that broaden the tested population to include paucibacillary and atypical presentations. The finding is broadly consistent with Khursheed et al., who documented substantial TB burden in a retrospective analysis from Muzaffarabad division of AJK,(2) and with a GeneXpert-based study from Mirpur division that reported a positivity rate of 17.6% among 580 tested patients,(3) suggesting that high TB prevalence is a consistent feature across AJK divisions rather than a localised phenomenon.
The high testing volume — nearly 25,000 specimens across 14 facilities — demonstrates substantial GeneXpert network capacity in AJK. However, the markedly uneven distribution of testing volume, with CMH Rawalakot contributing 15.7% of all tests and DHQ Haveli contributing only 2.9%, reflects the persistent geographic accessibility challenges in remote districts. The NTP AJK should consider reassessing testing capacity allocation toward lower-volume districts such as Haveli (n=719) and Neelum Valley (n=898), where low testing volumes may represent unmet diagnostic need rather than genuinely lower TB burden.(4)

Trace-Level Results and Paucibacillary Disease
A clinically and programmatically significant finding of this study was the detection of 492 Trace-level results, constituting 17.8% of all MTB-positive cases. The Trace category is unique to the GeneXpert MTB/RIF Ultra platform and represents very low bacterial loads — defined by cycle threshold values exceeding 28 — that are undetectable by conventional smear microscopy and were not consistently identified by the earlier Xpert MTB/RIF assay.(28) The Trace proportion observed in this study (17.8%) is higher than the 10–15% range reported in published Ultra validation studies from high-burden settings,(29) potentially reflecting delayed healthcare-seeking behaviour in geographically isolated communities, a relatively older patient demographic, or uncharacterised HIV co-infection rates in AJK.
Beyond their diagnostic significance, Trace results present an important programmatic challenge. The majority of the 513 non-interpretable RIF resistance readings in this study arose from Trace-level cases, creating a diagnostic gap in which M. tuberculosis is detected but drug resistance status cannot be determined by the Ultra assay alone. These patients are at particular risk of inappropriate treatment initiation — being started on standard first-line regimens without knowledge of their true drug susceptibility profile. The NTP AJK should establish a standardised clinical follow-up protocol for all Trace-positive cases, including reflex Line Probe Assay or liquid culture with drug susceptibility testing, to enable appropriate treatment selection for this vulnerable subgroup.(30)

Geographic Heterogeneity in TB Burden
The significant spatial variation in MTB positivity rates across AJK districts — ranging from 7.4% in Poonch to 16.9% in Jhelum Valley — is one of the most clinically important epidemiological findings of this study and mirrors the subnational heterogeneity in TB incidence documented across Pakistan, who reported district-level incidence estimates ranging from 110 to 462 per 100,000 inhabitants.(31) Jhelum Valley's positivity rate of 16.9% — the highest in AJK — despite its lowest absolute testing volume (n=968) is particularly noteworthy. This pattern most likely reflects delayed diagnosis due to geographic isolation and limited healthcare access, resulting in patients presenting with more advanced, higher-burden disease at the time of testing. Longitudinal studies and molecular typing of circulating strains would be required to distinguish between delayed diagnosis and genuinely higher transmission as explanations for this finding.
In terms of absolute case burden, Muzaffarabad district contributed the highest number of confirmed TB cases (n=554, 20.0% of all positives), consistent with its role as the territorial capital and primary referral centre for surrounding areas, as previously documented(11) Mirpur ranked second (n=502, 18.1%), and together these two districts accounted for 38.1% of all MTB-positive cases in AJK during 2025 — a concentration with direct implications for resource allocation within the NTP. The lower positivity rate in Poonch (7.4%) despite the highest testing volume (n=3,935) may reflect broader presumptive TB screening criteria at CMH Rawalakot — a military health facility — or genuinely better TB control in this district. The complete absence of RR-TB in five of ten districts warrants cautious interpretation, as it may reflect genuine low resistance prevalence, limited XDR testing coverage in those areas, or inadequate case detection rather than true absence of drug-resistant disease.

Rifampicin Resistance and Primary versus Acquired Drug Resistance
The overall RR-TB rate of 0.93% (95% CI: 0.61–1.42%) among RIF-interpretable results is lower than WHO-estimated national MDR-TB rates of approximately 4% among new cases and 17% among previously treated cases in Pakistan,(1) suggesting that AJK may harbour a comparatively lower rifampicin resistance burden than more urbanised Pakistani provinces. This finding is broadly consistent with previously published AJK data(2) and the Mirpur division study,(32) though direct comparison is limited by differences in study design, time period, and patient populations.

The most epidemiologically alarming finding within the RR-TB profile was that 13 of 21 confirmed RR-TB cases (61.9%) were new patients with no prior anti-TB treatment history — meeting the definition of primary rifampicin resistance and indicating active community transmission of already-resistant M. tuberculosis strains in AJK.(6) This pattern has fundamentally different programmatic implications from acquired resistance: it points to gaps in contact tracing and community-level infection control rather than simply treatment adherence failures. The remaining 8 cases (38.1%) represented acquired resistance among previously treated patients, consistent with the well-established mechanism of selective amplification of rpoB mutations during inadequate or interrupted treatment,(6) and highlights continued gaps in directly observed therapy implementation and treatment completion support across AJK's TB program.(19,20)

The striking concentration of RR-TB in Mirpur district — which accounted for 47.6% of all RR-TB cases despite contributing only 15.3% of total testing volume and recording an average overall positivity rate (13.1%) — warrants urgent molecular epidemiological investigation. This disproportionate burden of drug-resistant disease suggests the possibility of a localised cluster of drug-resistant M. tuberculosis transmission. Mirpur's unique demographic profile, characterised by exceptionally high rates of transnational migration to and from the United Kingdom, may contribute to importation of drug-resistant strains from higher-burden international settings — a hypothesis requiring confirmation through whole-genome sequencing of circulating strains to determine whether a clonal drug-resistant outbreak is occurring.(34)

Extended Drug Resistance Profile — GeneXpert MTB/XDR Analysis

The GeneXpert MTB/XDR findings represent the most clinically significant and novel contribution of this study. Among 18 RIF-resistant cases referred for XDR testing, isoniazid resistance was detected in 7 of 14 interpretable results (50.0%; 95% CI: 26.8–73.2%), confirming MDR-TB in these cases. This MDR-TB rate among RIF-resistant cases is consistent with WHO global estimates indicating that approximately 50% of RR-TB cases worldwide meet the full MDR-TB definition,(1) lending validity to our findings. Fluoroquinolone resistance was additionally detected in 5 of 14 interpretable results (35.7%; 95% CI: 16.3–61.2%) — a rate substantially higher than the WHO-estimated global FQ resistance prevalence of approximately 17% among MDR-TB cases,(1) suggesting a potentially higher pre-XDR-TB burden in AJK than national and global estimates would predict. Cases with combined RIF, INH, and FQ resistance meet the WHO 2022 definition of pre-XDR-TB, representing a therapeutic crisis requiring specialised treatment regimens not routinely available at district-level NTP facilities in AJK.(35)

The detection of isoniazid resistance in 5 of 26 interpretable RIF-sensitive cases (19.2%; 95% CI: 8.5–37.9%) and fluoroquinolone resistance in 6 of 22 interpretable RIF-sensitive cases (27.3%; 95% CI: 13.3–47.5%) is arguably the most clinically consequential finding of this entire study. These patients — who tested negative for rifampicin resistance by MTB/RIF Ultra and would routinely be classified as drug-sensitive TB — harboured isoniazid mono-resistance or fluoroquinolone resistance detectable only through reflex XDR testing. Without this additional molecular characterisation, all affected patients would have been initiated on standard first-line regimens containing isoniazid and fluoroquinolones as key therapeutic components, which would have been ineffective against their actual infecting strains. This carries a twofold consequence: individual treatment failure with risk of clinical deterioration and resistance amplification(22), and continued community transmission of drug-resistant strains in the absence of appropriate infection control. This finding provides compelling evidence for the expansion of GeneXpert MTB/XDR testing beyond confirmed RR-TB cases to include previously treated patients and those with clinical risk factors for drug resistance, irrespective of their RIF resistance status.(36)

Demographic Characteristics of RR-TB Cases

The near-equal sex distribution among RR-TB cases (male 52.4%, female 47.6%) diverges from South Asian studies reporting strong male predominance in drug-resistant TB(23), potentially reflecting the relatively small sample size (n=21) or genuine differences in exposure patterns in AJK's population. The mean age of 47.6 years and concentration of cases in the 50–59 years age group (38.1%) is consistent with global patterns of MDR-TB burden disproportionately affecting middle-aged adults — the population segment with the highest probability of prior TB treatment exposure and consequent acquired resistance.(1) The identification of one RR-TB patient aged 16 years — a new case meeting the definition of primary resistance — is particularly concerning as it confirms transmission of drug-resistant M. tuberculosis to the adolescent population, representing a sentinel finding that should prompt immediate contact investigation within the patient's household and school environment.

The exclusive presentation of all 21 RR-TB cases as pulmonary TB should not be interpreted as confirming the absence of extrapulmonary drug-resistant TB in AJK. Extrapulmonary specimens are less systematically referred for GeneXpert testing in routine NTP settings, and extrapulmonary drug-resistant disease likely represents a systematic ascertainment gap in this study's case identification.

Temporal and Seasonal Trends

Quarterly analysis revealed a peak in MTB positivity during Q2 (April–June 2025; 12.3%) with a progressive decline through Q3 (11.1%) and Q4 (9.9%). The Q2 peak may reflect several overlapping factors: increased post-winter healthcare-seeking behaviour as mountain communities gain improved access to health facilities following winter road closures; nutritional and physiological effects associated with Ramadan, which fell across Q1–Q2 of 2025 and has been associated with altered immune function and TB reactivation in susceptible individuals in some published analyses;(8) and spring-time population movement that may facilitate transmission across districts. The Q3 testing volume peak (n=6,553), despite a lower positivity rate (11.1%), likely reflects improved summer road accessibility enabling greater specimen transport from peripheral facilities through the STP and higher healthcare utilisation across the region.

The concentration of RR-TB cases in Q3 (n=7, 33.3% of annual RR-TB) is of particular epidemiological significance. A summer peak in drug-resistant TB detection may reflect increased population movement during this period — potentially importing drug-resistant strains from urban centres or cross-border contacts — or may represent a genuine transmission peak related to summertime crowding in communal settings such as agricultural worksites and religious gatherings. Importantly, the consistent identification of RR-TB across all four quarters confirms that drug-resistant TB is endemic rather than episodic in AJK, requiring sustained year-round vigilance and maintained diagnostic capacity [37].

Specimen Transportation Program and Diagnostic Equity

The Specimen Transportation Program demonstrated a critical equity contribution to TB case detection in AJK. Of 2,883 specimens transported from 32 peripheral health facilities across five districts, 256 (8.9%) tested positive for M. tuberculosis and 4 RR-TB cases were identified — the latter representing 19.0% of all annual RR-TB cases detected in AJK during 2025, despite STP samples comprising only 11.5% of total testing volume. This disproportionately high RR-TB yield from the STP network suggests that remote, STP-served communities may harbour a higher drug resistance burden than facility-based data alone would indicate, possibly reflecting higher rates of prior treatment, delayed diagnosis, or limited DOT supervision in geographically isolated settings. Without the STP, these 4 RR-TB patients would have faced significantly delayed or absent molecular diagnosis, with consequent prolonged community transmission of drug-resistant strains and delayed initiation of appropriate second-line therapy.(38)

The Q3 peak in STP transportation volume (n=1,016) compared to Q2 (n=407) is consistent with improved summer road accessibility in AJK's mountainous terrain, highlighting the seasonal constraint on diagnostic equity in this region. These findings collectively support the prioritised expansion of the STP network to additional peripheral facilities — particularly in Neelum Valley and Jhelum Valley, where geographic isolation is most pronounced and where the highest district-level TB positivity rate (16.9%) in this study was recorded.

Public Health and Programmatic Implications

The findings of this study generate five direct, evidence-based recommendations for TB control policy in AJK. First, the predominance of primary rifampicin resistance (61.9%) among new RR-TB cases demands immediate intensification of contact investigation around every confirmed RR-TB index case, with systematic GeneXpert screening of all household contacts to interrupt ongoing transmission chains. Second, the high rates of isoniazid mono-resistance and fluoroquinolone resistance detected by XDR analysis among RIF-sensitive cases provide compelling evidence for the expansion of GeneXpert MTB/XDR testing as a reflex investigation for all previously treated patients and clinical treatment failures, irrespective of RIF resistance status. Third, the disproportionate concentration of 47.6% of all RR-TB cases in Mirpur district justifies targeted molecular surveillance through whole-genome sequencing to determine whether a clonal drug-resistant outbreak is occurring and to guide a focused outbreak response. Fourth, the 492 Trace-positive cases, identifiable exclusively through the Ultra platform, validate continued deployment of GeneXpert MTB/RIF Ultra across AJK and highlight the urgent need for standardised clinical management protocols for Trace results within the NTP framework. Fifth, the STP program's disproportionately high RR-TB yield from peripheral facilities supports its prioritised expansion and strengthening, particularly toward Neelum Valley and Jhelum Valley, to ensure geographic isolation does not constitute a barrier to molecular TB diagnosis.

Limitations

Several limitations should be acknowledged when interpreting these findings. First, the facility-level aggregate nature of GeneXpert MTB/RIF Ultra data precluded individual patient-level demographic analysis of TB positivity predictors, individual data were available only for the 21 confirmed RR-TB cases, limiting the scope of risk factor analysis across the full study population. Second, GeneXpert MTB/XDR testing was performed at a single facility and covered only 50 of 2,771 MTB-positive cases, representing a highly selected and potentially non-representative subgroup, the true prevalence of isoniazid, fluoroquinolone, and aminoglycoside resistance across AJK may differ substantially from these estimates. Third, the clinical criteria for selecting RIF-sensitive cases for XDR testing were not systematically documented, introducing potential selection bias that limits the

generalisability of RIF-sensitive resistance findings. Fourth, HIV status was not recorded in NTP laboratory registers, precluding assessment of TB-HIV co-infection rates(25) and their contribution to Trace positivity. Fifth, aminoglycoside resistance data were unavailable, preventing complete pre-XDR-TB and XDR-TB classification. Sixth, this study covers only a single calendar year — longitudinal multi-year data would be required to confirm whether the temporal trends, seasonal patterns, and geographic resistance clusters described herein represent stable epidemiological features or transient fluctuations. Seventh, as a retrospective study dependent on routine NTP documentation, completeness and accuracy of data were constrained by existing record-keeping practices, and the possibility of under-reporting cannot be excluded.

Conclusion

In conclusion, this study establishes the first comprehensive molecular epidemiological baseline for tuberculosis across all ten districts of Azad Jammu and Kashmir using a two-assay GeneXpert diagnostic cascade during 2025. The overall MTB prevalence of 11.09% . exceeding national programmatic averages , combined with evidence of active primary drug-resistant TB transmission in the majority of RR-TB cases, a disproportionate concentration of drug-resistant disease in Mirpur district, and previously unrecognised isoniazid and fluoroquinolone resistance in RIF-sensitive cases detected exclusively through XDR analysis, collectively characterise AJK as a region with a molecularly complex and inadequately characterised drug-resistant TB burden. These findings provide actionable, district-specific evidence to guide the NTP AJK in prioritising contact investigation, expanding GeneXpert MTB/XDR testing coverage, strengthening the Specimen Transportation Program, and targeting molecular surveillance resources toward high-burden districts , particularly Mirpur and Muzaffarabad. Future studies incorporating whole-genome sequencing, Line Probe Assay for complete first-line drug susceptibility profiling, and multi-year longitudinal design would substantially advance understanding of M. tuberculosis transmission dynamics and evolving drug resistance patterns in this strategically important and epidemiologically undercharacterised region. This study establishes the first comprehensive, region-wide molecular epidemiological baseline for tuberculosis across Azad Jammu and Kashmir (AJK) utilizing a two-assay GeneXpert diagnostic cascade. The findings reveal a substantial TB burden (11.1% positivity) that exceeds national programmatic averages, compounded by a complex and previously under-recognized landscape of drug resistance. Most alarmingly, the predominance of primary rifampicin resistance among new cases (61.9%) signals active, ongoing community transmission of resistant strains, while the disproportionate concentration of RR-TB in Mirpur district highlights a potential localized transmission hotspot requiring immediate epidemiological investigation.

Critically, the application of the GeneXpert MTB/XDR assay unveiled a dangerous diagnostic blind spot: significant rates of isoniazid mono-resistance and fluoroquinolone resistance persisting within cases initially classified as rifampicin-sensitive by standard Ultra testing. Without reflex XDR testing, these patients would have been inappropriately managed with standard first-line regimens, risking individual treatment failure, resistance amplification, and continued community transmission. This underscores the inadequacy of relying solely on rifampicin resistance as a proxy for comprehensive drug susceptibility in this region.

To mitigate this evolving therapeutic crisis, the National TB Control Programme (NTP) in AJK must adopt differentiated, evidence-based strategies. Priority actions include: (1) expanding reflex MTB/XDR testing to previously treated patients and clinical non-responders, regardless of initial RIF status; (2) intensifying active case finding and contact tracing around all new RR-TB index cases, particularly in high-burden districts like Mirpur and Muzaffarabad; (3) formalizing standardized clinical management protocols for "Trace" positive results to prevent diagnostic ambiguity; and (4) scaling up the Specimen Transportation Program to ensure equitable diagnostic access in geographically isolated districts such as Neelum and Jhelum Valley. Future research incorporating whole-genome sequencing (WGS) and multi-year longitudinal surveillance is essential to elucidate transmission dynamics, confirm suspected clonal outbreaks, and guide the next generation of targeted TB elimination efforts in this strategically vital region.

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