ORIGINAL ARTICLE

Evaluation Of Phenotypic and Genotypic Methods for Detection of Multidrug Resistant Tuberculosis in Pakistan

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ABSTRACT

Objective: To evaluate the phenotypic and genotypic approaches for the diagnosis of drug-resistant *Mycobacterium Tuberculosis*.

Study Design: A cross-sectional study.

Place and Duration of Study: This multicenter study was conducted over a period of six months, from April 01, 2025, to September 30, 2025, in collaboration with several healthcare and academic institutions across Pakistan. The study activities were carried out in the Biosafety Level 3 (BSL-3) Laboratory at the Department of Microbiology, Health and Population Department, Lahore; Tehsil Headquarters (THQ) Hospital Jand, district Attock; Health Services Academy, Islamabad; Islamabad Medical and Dental College, Barakaho; Metropole Laboratories, Islamabad; and Sindh Medical College, Karachi.

Materials and Methods: Eighty-Seven culture-positive samples of *Mycobacterium Tuberculosis* (MTB) isolates were evaluated for susceptibility to first-line anti-tuberculosis medications such streptomycin, isoniazid, ethambutol, and rifampicin, by using the Mycobacteria Growth Indicator Tube (MGIT) 960 system. The GeneXpert for MTB was used for its rapid detection and rifampicin resistance. Multiplex polymerase chain reaction (PCR) assays were optimized to amplify mutations in the *rpoB* gene associated with rifampicin resistance, the *katG* gene associated with isoniazid resistance, and the *embB* gene associated with ethambutol resistance. The amplified PCR products were analyzed by agarose gel electrophoresis to determine the banding patterns associated with multidrug-resistant (MDR) and extensively drug-resistant (XDR) MTB isolates.

Results: Among the 84 MTB isolates, 30 samples were single-drug resistance (SDR), while 4 samples were identified as MDR. Among the SDR isolates, resistance to isoniazid was the most common (n = 24), followed by ethambutol (n = 3) and rifampicin (n = 2). Three non-MTB samples (negative, positive, and normal controls) were included as controls. Additionally, 50 samples were identified as Mycobacteria Other Than Tuberculosis (MOTT).

Multiplex PCR analysis included the following diagnostic performance parameters for MDR: sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and diagnostic accuracy (DA) and it demonstrated 66.67%, 100%, 33.33%, 71.43%, and 80% for multidrug resistance; 66.67%, 100%, 50%, 75%, and 85.71% for isoniazid resistance; 80%, 96%, 50%, 84.50%, and 100% for rifampicin resistance; and 50%, 75%, 100%, 80%, and 80% for ethambutol resistance, respectively.

Conclusion: Genotypic methods for diagnosing drug-resistant MTB were faster than phenotypic methods to address important public health challenges in countries like Pakistan.

Key Words*: Genotypic, Mycobacterium tuberculosis, Phenotypic*.

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Introduction

Mycobacterium tuberculosis (MTB) is one of the major health problems with significant morbidity and mortality worldwide.[1] In 2020, the World Health Organization (WHO) published recommendations for tuberculosis (TB) diagnosis and treatment. For developing and low-resource nations alike, TB presents a serious problem. Approximately 25% of the world's population, or over 1.7 billion individuals, have MTB infections.[1,2] Pakistan is ranked fifth for TB and fourth for multi-drug-resistant

tuberculosis (MDR-TB) in the WHO's global report.[1,3-5] Laboratories and clinical radiological studies are used to diagnose TB. However, latent TB was identified by the tuberculin skin test (TST) and the interferon gamma release assay (IGRA). Culture-based testing, which yields phenotypic information, and molecular testing, which yields genotypic information, are two laboratory methods for determining drug susceptibility. The most accurate method now available for diagnosing drug-resistant tuberculosis is culture-based testing, which can take up to a month to complete. Drug resistance can be identified by comparing growth on the drug-containing media with growth on the control medium.[6]

Molecular testing yields results faster; they may now be acquired in a few hours. In the absence of a definitive culture-based Direct Susceptibility test (DST) these tests are useful in assisting with early therapeutic decisions. [7] The two broad categories into which molecular tests may be separated are sequencing-based assays and probe-based (non-sequencing) testing. Probe-based testing is also referred to as amplification assays for nucleic acids. Drug resistance genes can be found utilizing specialized nucleic acid amplification tests (NAAT) that amp up a certain nucleic acid sequence that can be identified with a nucleic acid probe. Assays that rely on sequencing are still being developed and have not been approved globally.[4,8]

The FDA has approved the use of two NAAT test platforms in the United States: The amplified Mycobacterium tuberculosis direct (MTD) test and the Xpert MTB/RIF test. The amplified MTD test is approved for respiratory specimens from people who have suspected TB, regardless of whether the specimens are smear-positive or smear-negative.[9] There are a few studies available which combine genotypic and phenotypic approaches for MTB detection; nevertheless, genotypic approaches aid in the prompt and precise identification of MTB and medication resistance for the patient's benefit.[9]

Therefore, the aim of this study was to evaluate the phenotypic approaches of Mycobacterium Growth Indicator Tube (MGIT) Culture and genotypic approaches (Gene Xpert and Multiplex PCR) for the diagnosis of drug-resistant MTB.

Materials and Methods

A cross-sectional study was carried out in BSL-3 at the Department of Microbiology, Health and population department Lahore, in collaboration with Shaheed ed Uzair Mehmood Tehsil Headquarters (THQ) Hospital Jand district Attock; Health services Academy, Islamabad; Islamabad Medical and Dental College, Barakahu; Metropol Hospital, Islamabad; Sindh Medical Collage Karachi. This multicenter study was conducted over a period of six months, from April 01, 2025 to September 30, 2025.

Using the WHO sample size calculator, the sample size was determined with a 95% confidence level, a 5% margin of error, and a reported sensitivity of 17.33% (GenoType) performance compared to phenotypic direct susceptibility test as reference technique). The sample size was 87 and the method of sampling was sequential, non-probability sampling.

Multidrug resistant (MDR) and single-drug resistant (SDR) samples were chosen for multiplex PCR and pulse field gel electrophoresis in Pakistan after MTB sample collection and Kinyoun Staining.

All smear-positive pulmonary and extrapulmonary drug-resistant MTB isolates, including those that were SDR and MDR were included in the study. We have also included Mycobacterium Other Than Tuberculosis (MOTT) and MTB isolates that are resistant to antituberculosis medications as a control. After the bar code was scanned, every MGIT was put into the system to be incubated. After an infected material produced a culture that was positive in the MGIT system, Digestion, decontamination, homogenization, and concentration were all aided by pretreating smear-positive only specimens using the conventional sodium hydroxide Nacetyl cysteine procedure.[10]

One step in the procedure was applying Kinyoun stain to a smear.

Following WHO guidelines, the smear's grade was established based on the quantity of acid-fast bacilli (AFB) visible under a microscope. A BACTEC MGIT 960 system was injected with a processed smear-positive sample to culture the MTB isolate. Culture media (7H9 broth, 7mL) was put into the barcoded MGIT.[10]

A growth supplement was provided to encourage the growth of MTB, and an antibiotic combination

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known as polymyxin, amphotericin, nalidixic acid, trimethoprim, azlocillin (PANTA) was added to stop other bacteria from infecting the MGIT tubes. Kinyoun staining and the BD MGIT TB identification test, a fast chromatographic immunoassay for MTB antigen detection, are used to confirm it. The MOTT were now identified and eliminated. Before the direct DST setup, 15 milliliters of SIRE supplements (Becton Dickinson Diagnostic Systems, Sparks, MD) were added to the lyophilized PANTA and thoroughly mixed. Two lyophilized drugs, isoniazid and rifampicin (the same ones used in MGIT), were fully blended and reconstituted using four milliliters of sterile deionized (DI) water. Regarding the direct Drug Susceptibility for every specimen, a set of three MGIT tubes was constructed. Three tubes had labels: one for growth control (GC), one for rifampicin and one for isoniazid. The PANTA-SIRE supplement combination (800µL) was added to the designated MGIT tubes. The appropriate drugs were then put into tubes with labels. In the tube designated rifampicin, 100µL of reconstituted rifampicin (1.0µg/mL) and 100µL of isoniazid drugs were added. The final concentration (0.1µg/mL) was added to the tube labeled isoniazid. Then, 500µL of the thoroughly mixed smear-positive processed material was added to each of the two medicine tubes. 500µL of water or sterile saline was diluted 1:10 with the material that has been treated prior to its introduction into the GC tube. The growth control, rifampicin, and isoniazid tubes were placed in the set carrier after being placed within the instrument. In the set carrier, the GC tube was always the first tube. When growth unit value was ≥ 400, the GC test was deemed finished. After scanning, the MGIT tubes were taken out of the device, and an inventory report was made. Any test result that had a growth unit (GU) value below 100 was deemed "susceptible."

The outcome was considered "resistant" if the GU value was more than or equal to 100. Recorded were the GU values. The growth was deemed insufficient if, after 21 days, the GU value in the control tube did not reach 400. On the other hand, GU above 400 prior to four days was considered a sign of contamination or over-inoculation. After thoroughly mixing with a pipette, 250µL of cell lysis solution was transferred to a 1.5µL Eppendorf tube containing 50µL of MTB culture isolates for DNA extraction. The tube was then heated to 65°C for 15 minutes. Following cell lysis, samples were allowed to cool to room temperature before being vortexed and mixed with 100µL of protein precipitation solution. The samples were centrifuged at 15,000rmp for 5 minutes after being allowed to reach room temperature. The DNA-containing supernatant and 250µl of 100% isopropanol were carefully transferred into a clean Eppendorf tube following the protein precipitation stage, and the mixture was gently agitated by inversion.

For three minutes, the samples were centrifuged at 15,000rmp. After discarding the supernatant, the particle was cleaned with 70%, and the data was examined using Medcalc. A 2x2 contingency table was used to calculate the sensitivity, specificity, positive and negative predictive values, and diagnostic accuracy of genotypic and phenotypic testing.

Results

A total of 54 samples that satisfied the requirements were used to evaluate the multiplex PCR procedure for drug-resistant MTB strains. Details of the primer used for DNA extraction were mentioned in Table I and II.

Table I: The MTB Sample Genotyping Primers and PCR (n=87)

Anti-TB Drugs	Targets site	Primers	Tm (°C)	PS
RIF	*rpoB* (530)	F: GATCAGTTGATCAACATC R: TACGGCGTTTCGATGAAC	55	305
INH	*katG* (315)	F: GGCATGAGCGTTACAC R: CCCTCTGGCGGTGTATT	55	458
EMB	*embB* (344)	**F:** CGCGAACCCTGGTGGCTTC **R:** GGCGTTCACAACGACGACG	55	306

RIF: Rifampicin, INH: Isoniazid, EMB: Ethambutol, Tm: Temperature, PS: Product Size, Ref: Reference.

Table II: Multiplex PCR Reaction Mixture

Reagents	Concentration	Volume
Distilled water		16.1 µl
Forward primers for the genes *Kat G, emb B* , and *Rpo B*	1µM	3µ1
Reverse primers for *rpo B, Kat G*, and *emb B* genes	1µM	3µl
Magnesium chloride ($MgCl_2$)	2.5µM	2.5µl
Buffer without $MgCl_2$	2µM	2µl
dNTPS	0.5µM	0.5µl
Taq polymerase	0.5µM	0.5µl
DNA	1-5 ng	1.4µl
Total		25-27 µl

Table III: **Genotypic Values and Resistance Pattern of MDR, INH, RIF & EMB (n = 87)**

Resistance Pattern	Genotypic testing (Multiplex PCR)	Phenotypic testing (MGIT CULTURE) R	S	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	DA (%)
MDR	**R**	4	0	66.67%	100%	100%	33.33%	71.43%
	S	2	1					
RIF	**R**	2	0	66.67%	100%	100%	50%	75%
	S	1	1					
INH	**R**	24	1	85.71%	80%	96%	50%	84.50%
	S	4	4					
EMB	**R**	3	1	100%	50%	75%	100%	80%
	S	0	1					

MDR: Multi-drug resistant, INH: Isoniazid, RIF: Rifampicin, EMB: Ethambutol, PPV: Positive predictive value, NPV: Negative predictive value, DA: Diagnostic accuracy

Three samples used control positive and negative and normal control as non-MTB samples, whereas four of the 54 MTB samples were multidrug resistant and thirty were single drug resistant (SDR). Two of the thirty SDR were resistant to rifampicin, twenty-seven to isoniazid, and three to ethambutol. According to multiplex PCR, the genotypic values of MDR were 66.67 %, 100 %, 33.33 %, 71.43 %, PPV, NPV, and DA; rifampicin were 66.67 %, 100 %, 100 %, 50%, 75.00 %; isoniazid were 85.71 %, 80 %, 96 %, 50 %, 84.50 %; and ethambutol were 100 %, 50 %, 75 %, 100 %, and 80 %, respectively. (Table III)

Using primer pairs for the rifampicin (*rpoB*), isoniazid (*katG*), and ethambutol (*embB*) genes, multiplex PCR created a distinctive banding pattern on a 1% agarose gel stained with ethidium bromide. Resistance to rifampicin is specified by a single 305 bps band. Resistance to isoniazid is specified by a single 458 bps band. Resistance to ethambutol is specified by a single band of 306 bps. The resistance to rifampicin and isoniazid is defined by two bands, 305 bps and 458 bps. Two bands, measuring 458 bps and 306 bps, respectively demonstrate the resistance to isoniazid and ethambutol. Lane M is a 100 and DNA as seen in figures 2; Lanes 1 through 8 are SDR (isoniazid), SDR (rifampicin) and MDR (rifampicin and isoniazid) in Lanes 3-5 and 7, as shown in Figure 1 & 2

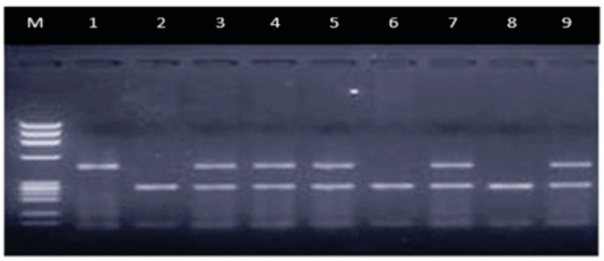

Figure 1: Gel Electrophoresis to Identify the Resistance Pattern of MDR *RpoB* Rifampicin and Isoniazid for *KatG* Genes

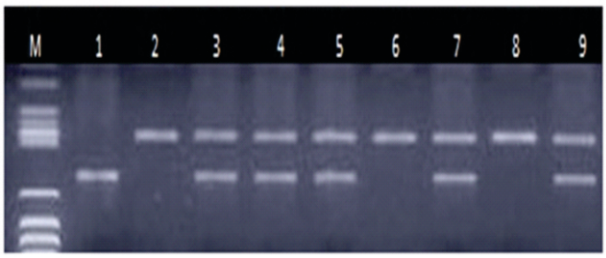

Figure 2: Resistance Pattern of the Extensive Drug Resistant *RpoB, KatG, InhA,* And *EmbA* Genes in Gel Electrophoresis

Discussion

Humans have had TB for thousands of years. For TB, drug susceptibility testing and culture-based diagnostic techniques remain the gold standard. The results of these techniques take at least six to eight weeks. On the other hand, molecular techniques show promise for a quick and precise diagnosis of drug-resistant TB. Most of the newly created molecular techniques are costly, need a high level of quality control, and are unavailable in distant regions with high illness incidence. Furthermore, only rifampicin resistance may be diagnosed using the WHO-approved GeneXpert. Due in large part to delayed diagnosis, drug-resistant tuberculosis is becoming more common in Pakistan. To quickly diagnose drug-resistant tuberculosis in distant places, a simple, affordable molecular technique is needed.

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Rifampicin resistance may be detected via multiplex PCR with varying sensitivity depending on the location. Sensitivity and specificity in our analysis were found to be 100% equivalent to Chinese data. [11] The bulk of rifampicin resistance in MTB is caused by mutations in the *rpoB* gene. [12] According to earlier findings from Pakistan, most isolates had a mutation at codon S531L of the *rpoB* gene. [12]

Finding a mutation within the rifampin drug resistant of *rpoB* is crucial for the quick identification of MTB isolates. This is supported by the following lines of evidence: first, the molecular mechanism of rifampin resistance is the most fully understood of the anti-tuberculosis drugs currently in the market. It has been found that 95% or more of rifampicin-resistant isolates had a second mutation inside the rifampicin drug resistant of *rpoB*. In our investigation, 17 (26%) isolates with rifampicin resistance were found to have a mutant band in multiplex PCR 100% diagnostic accuracy.

Genotypic testing for isoniazid resistance is much more challenging than rifampin. First, isoniazid resistance is linked to changes in at least three genes: *katG, inhA*, and *ahpC.* Second, a mutation in the regulatory regions of either *ahpC* or *katG*. Third, whereas certain *katG* mutations may not provide resistance to clinically relevant concentrations of isoniazid others are linked to low level resistance (deletion or S315T). Fourth, focusing on codons that are most modified globally may miss local strain populations and/or epidemic strains, which may be reflected in the relative frequency of a certain mutation. Fifth, it is unclear how the overexpression of the *ahpC* gene and *inhA* are related. [13] The 458 bp section of *katG* that includes codon 315 was employed in our investigation. 28 (42%) of the 39 isoniazid-resistant isolates detected in MGIT 960 had *katG* mutations. On the other hand, the target region of the *katG* gene included no mutations in the remaining 11 (17%). Threonine replacing serine amino acid 3 was, as expected, the most common *katG* modification.

We used a 458 bp segment of the *kat G* gene for INH and a 305 bp segment of *rpoB* for rifampicin in our investigation of MDR-TB. The 18 MDR isolates were identified using the MGIT 960 technique; 11 (17%) of these isolates showed characteristic banding patterns of concurrent *rpoB* and *katG* mutations in multiplex PCR followed by gel electrophoresis. In China, a similar resistance pattern was discovered by a similar experiment on MDR MTB. [14] This study's primary goal was to determine how much time MTB genotypic testing saves in comparison to phenotypic testing. [15] Genomic testing saved a maximum of 8 to 13 days (depending on the genotype) in comparison to MGIT culture or phenotypic test. The most plausible reason could be that the study was limited to one location and that the sample size was appropriately sized. Doctors can begin early therapy and prevent the spread of drug-resistant TB isolates thanks to this time-saving technique.

Overall, our findings suggest that genotypic testing is a reliable method, demonstrating a high level of agreement with MGIT culture and drug susceptibility testing. Given its potential for rapid detection, genotypic testing may serve as a valuable complementary diagnostic approach for TB and drug resistance in the Pakistani population.

Our research had two limitations. Initially, specimens that were tested positive for smears were used. Afterwards, just 21 percent of the isolates had multidrug resistance.

Conclusion:

Genotypic methods for diagnosing drug-resistant MTB were faster than phenotypic methods to address important public health challenges in countries like Pakistan.

Disclaimer: The research is conducted by the author using available resources with no funding. The opinions and content are solely the responsibility of the author and not the journal's editors or publisher.

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CONFLICT OF INTEREST

Authors declared no conflicts of Interest.

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DATA SHARING STATEMENT

The data that support the findings of this study are available from the corresponding author upon request.