ORIGINAL ARTICLE

Comparative evaluation between the genotypic and phenotypic methods for detection of drug-resistant *Mycobacterium tuberculosis*

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ABSTRACT

Background: Tuberculosis (TB) is one of the major health problems with significant morbidity and mortality worldwide. According to world health organization in 2020 published guidelines for diagnosis and treatment of TB. To compare between genotypic and phenotypic methods for detection of drug resistant *Mycobacterium tuberculosis*.

Material and Methods: This cross-sectional validation study was conducted from January to September 2024 in BSL-3 laboratories of Islamabad Diagnostic Center, Izzat Ali Shah Hospital, and POF Hospital Wah Cantt, Pakistan. A total of 87 culture-positive *Mycobacterium tuberculosis* (MTB) isolates were tested for susceptibility to Rifampicin, Isoniazid, Ethambutol, and Streptomycin using the MGIT 960 system and GeneXpert. DNA was extracted with the Gentra System kit, and multiplex PCR targeting *rpoB*, *katG*, and *embB* genes was performed. Gel electrophoresis was used to compare banding patterns of MDR and XDR MTB.

Results: Out of 87 MTB samples 4 were multidrug resistant (MDR) and 30 were Single drug resistant (SDR). Among single drug resistant 2 were Rifampicin resistant, 24 were isoniazid resistant ,3 were ethambutol resistant. 3 samples use control (negative, positive and normal) non-MTB samples. and 50 samples were detected as MOTTS, The Sensitivity, Specificity, PPV, NPV and DA of MDR, Rifampicin, Isoniazid and Ethambutol were66.67%, 100.00%,33.33%, 71.43%,66.67%, 100.00%, 100.00%, 50.00%, 75.00%,85.71%,80.00%,96.00%, 50.00%,84.50%, ,100.00%, 50.00%,75.00%, 100.00%and 80.00% respectively genotypically by (multiplex PCR).

Conclusion; In this study genotypic methods for detection of drug resistant MTB were rapid as compare to phenotypic methods for timely and active management.

Keywords: Genotypic, *Mycobacterium* tuberculosis, Phenotypic.

BACKGROUND

Tuberculosis (TB) is one of the major health problems with significant morbidity and mortality worldwide. According to world health organization in 2020 published guidelines for diagnosis and treatment of TB.[1] TB poses a significant challenge for countries with limited resources and those in development. More than 1.7 billion people (about 25% of the world population) are infected with *Mycobacterium tuberculosis* (MTB).[2,3] According to the global report by WHO, Pakistan ranked fifth in the TB and fourth in MDR-TB (Multi drug resistant tuberculosis).[4,5] To put it succinctly, laboratory and clinical radiological studies are used to diagnose tuberculosis. However, the tuberculin skin test (TST) and the interferon gamma release assay (IGRA) were used to check for latent TB.[6] Two laboratory techniques for drug susceptibility testing include molecular testing, which gives genotypic information, and culture-based testing, which provides phenotypic information. The most reliable method for diagnosing drug-resistant tuberculosis is currently culture-based testing, which can take up to a month to complete. To ascertain whether drug resistance exists, growth on the drug-containing media is compared to growth on the control medium.

Molecular testing yields answers faster—they may now be obtained in a matter of hours. These tests are helpful in helping make early therapy decisions until a conclusive culture-based DST becomes available.[7] Probe-based (non-sequencing) testing and sequencing-based assays are the two general categories into which molecular assays may be divided. probe-based testing,

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often known as nucleic acid amplification tests. By amplifying a specific nucleic acid sequence that can be recognized using a nucleic acid probe, certain NAA tests are able to detect genes that encode drug resistance. Assays based on sequencing are still under research and have not received worldwide approval.[8] The FDA has authorized the use of two NAA test platforms in the United States: the Xpert MTB/RIF test and the amplified *Mycobacterium* tuberculosis direct (MTB) test. For respiratory specimens from individuals with suspected tuberculosis, whether smear-positive or smear-negative, the amplified MTD test is authorized.[9] Rationale of study was integrating both genotypic and phenotypic methods in MTB detection but genotypic methods help accurate and timely detection of MTB detection and prise detection of drug resistance for the benefit of patient.

The objective of our study to compare between genotypic methods (Gene Xpert and Multiplex PCR) and phenotypic method *Mycobacterium* Growth Indicator Tube Culture (MGIT) for detection of drug resistant *Mycobacterium tuberculosis*.

MATERIAL AND METHODS

This study was carried out in BSL-3 at the department of microbiology, Islamabad diagnostic center (MGIT Culture), Izzat Ali Shah Hospital (MTB sample collection and kinyoun Stain) POF hospital Wah Cantt (MDR and SDR samples were selected for Multiplex PCR and pulse field gel electrophoresis) Pakistan. It was a cross-sectional validation study. The duration of our study was 9 months, as mention in IRB from 1st January to 30th September 2024. Non-probability, consecutive sampling technique was used. Sample size was calculated using WHO sample size calculator taking confidence level 95%, margin of error 5%, reported Sensitivity 17.33%10 (GenoType MTBD Rplus performance compared to phenotypic drug susceptibility testing (DST) as reference method.). The estimated sample size came out to be 87 participants.

The investigation included all smear-positive pulmonary and extrapulmonary drug-resistant MTB isolates such as Multidrug Resistant (MDR) and Singler Drug Resistant (SDR). As a control, we have also included MTB isolates that are resistant to antituberculosis drugs patient's repeated isolates and *Mycobacterium* other than TB (*Mycobacterium* other than tuberculosis) All MGIT tube was then placed in the system for incubation after bar code reading. Once an inoculated specimen yielded positive culture in MGIT system, Pretreating smear-positive only specimens with the standard sodium hydroxide Nacetyl cysteine method helped with digestion, decontamination, homogenization, and concentration.

Staining a smear with kinyoun stain was part of the process. The amount of acid-fast bacilli (AFB) seen under a microscope determined the grade of the smear, which was determined by following WHO recommendations. In order to cultivate the MTB isolate, a processed smear positive material was injected onto a BACTEC MGIT 960 system. The mycobacterial growth indicator tube (MGIT) barcoded was injected with culture medium (7H9 broth, 7mL). To promote the development of MTB, a growth supplement was supplied, and to prevent other bacteria from contaminating the MGIT tubes, an antibiotic combination called PANTA was introduced. It is confirmed by kinyoun staining and by BD MGIT TB identification test, which is a rapid chromatographic immunoassay for MTB antigen detection. The MOTT were now recognized and disposed of Lyophilized PANTA was reconstituted with 15 milliliters of SIRE supplement (Becton Dickinson Diagnostic Systems, Sparks, MD) and properly mixed prior to direct DST setup. INH and RIF, two lyophilized medications (the same as those used in MGIT), were reconstituted with 4 milliliters of sterile deionized (DI) water and well mixed. For the direct DST, a set of three MGIT tubes was constructed for each specimen. Three tubes had labels: one for INH, one for GC, and one for RIF. The 800µL PANTA-SIRE supplement mixture was added to the MGIT tubes that had been labeled. The appropriate medications were then put to tubes with labels. 100µL of INH medication (0.1µg/mL final concentration) was added to the tube labeled INH, and 100µL of reconstituted RIF (1.0µg/mL) was put to the tube labeled RIF. Next, 500µL of the well-blended smear positive processed material was introduced into each of the two tubes holding the medication. 500µL of sterile saline or water was diluted 1:10 with the processed material before being introduced to the GC tube. After being inserted into the instrument, the GC, INH, and RIF tubes were positioned in the set carrier. The GC tube was always the first tube in the set carrier. The test was considered completed for GC when the growth unit (GU) value was ≥400. At this point, an

inventory report was created and the MGIT tubes were removed from the apparatus after scanning. When the GU value was less than 100, the test result was classified as "susceptible. "For the GU value ≥100, the result was considered as "resistant". The values of GU were noted If after 21 days the GU value of the control tube did not rise to 400, the growth was considered inadequate. GU reaching 400 before 4 days, on the other hand, was seen as contamination or over inoculation. After thoroughly mixing with a pipette, 250µL of cell lysis solution was added to a 1.5µL Eppendorf tube containing 50µL of MTB culture isolates for DNA extraction. The tube was then heated at 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. After bringing the samples to room temperature, a 3-minute 15000g centrifugation was performed. Following the protein precipitation stage, the DNA containing supernatant was carefully poured into a clean Eppendorf tube together with 250µl of 100% isopropanol, and the mixture was gently mixed by inversion. The samples underwent a 5-minute, 15,000-g centrifugation. After discarding the supernatant, the particle was cleaned with 70%.

Data was analyzed by medcalc. 2×2 contingency table was used to calculate sensitivity, specificity, positive predictive value, negative predictive value and diagnostic accuracy of genotypic and phenotypic testing.

RESULTS

To assess the multiplex PCR process for drug resistant MTB strains, 54 samples in total that met the criteria. For DNA extraction primer details were mention in Table I and II.

Out of 54 MTB samples 4 were multidrug resistant (MDR) and 30 were Single drug resistant (SDR) 3 samples use control positive and negative and normal control as non-MTB samples. Out of 30 SDR 2 were rifampicin resistant, 27 were isoniazid resistant and 3 were as shown in figure III and IV**.** The Sensitivity, Specificity, PPV, NPV and DA of MDR were66.67%, 100.00%,33.33%, 71.43%, Rifampicin were 66.67%, 100.00%, 100.00%, 50.00%, 75.00%, Isoniazid were 85.71%, 80.00%, 96.00%, 50.00%, 84.50%, and Ethambutol were 100.00%, 50.00%, 75.00%, 100.00% and 80.00% respectively genotypically by (multiplex PCR**)** as shown in table III.

A characteristic banding pattern produced by multiplex PCR employing primer pairs for the RIF (rpoB), INH (katG), and EMB (embB) genes on a 1% agarose gel sta ined with ethidium bromide.A single 305 bps band spe cifies resistance to RIF.A single band of 458 bps specif ies resistance to INH.A single band of 306 bps specifie s resistance to EMB.Two bands, 305 bps and 458 bps, define the resistance to RIF and INH.There are two ban ds that show the resistance to INH and EMB, measurin g 458 bps and 306 bps,respectively.Lanes 1 through 8 a re SDR (INH), SDR (RIF), and MDR (RIF and INH) i n Lanes 3-5, 7; Lane M is a 100 bp DNA ladderas shown in Figures-III and IV.

Table-I: The MTB sample genotyping primers and PCR parameters.

Anti-TB Drugs	Targets site	Primers	Tm (°C)	PS
Rifampicin	*rpo B* (530)	F: GATCAGTTGATCAACATC R; TACGGCGTTTCGATGAAC	55	305
Isoniazid	kat G (315)	F: GGCATGAGCGTTACAC R: CCCTCTGGCGGTGTATT	55	458
Ethambutol	*emb B* (344)	**F:** CGCGAACCCTGGTGGCTTC **R:** GGCGTTCACAACGACGACG	55	306

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 (MgCl2)	2.5µM	2.5µl
Buffer without MgCl2	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: Statistical analysis of genotypic and phenotypic testing.

Resistance Pattern	Genotypic testing (multiplex PCR)	Phenotypic testing (MGIT CULTURE) R	S	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	DA (%)
MDR	**R**	4	0	66.67%	100.00%	100.00%	33.33%	71.43%
	S	2	1					
RIF	**R**	2	0	66.67%	100.00%	100.00%	50.00%	75.00%
	S	1	1					
INH	**R**	24	1	85.71%	80.00%	96.00%	50.00%	84.50%
	S	4	4					
EMB	**R**	3	1	100.00%	50.00%	75.00%	100.00%	80.00%
	S	0	1					

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

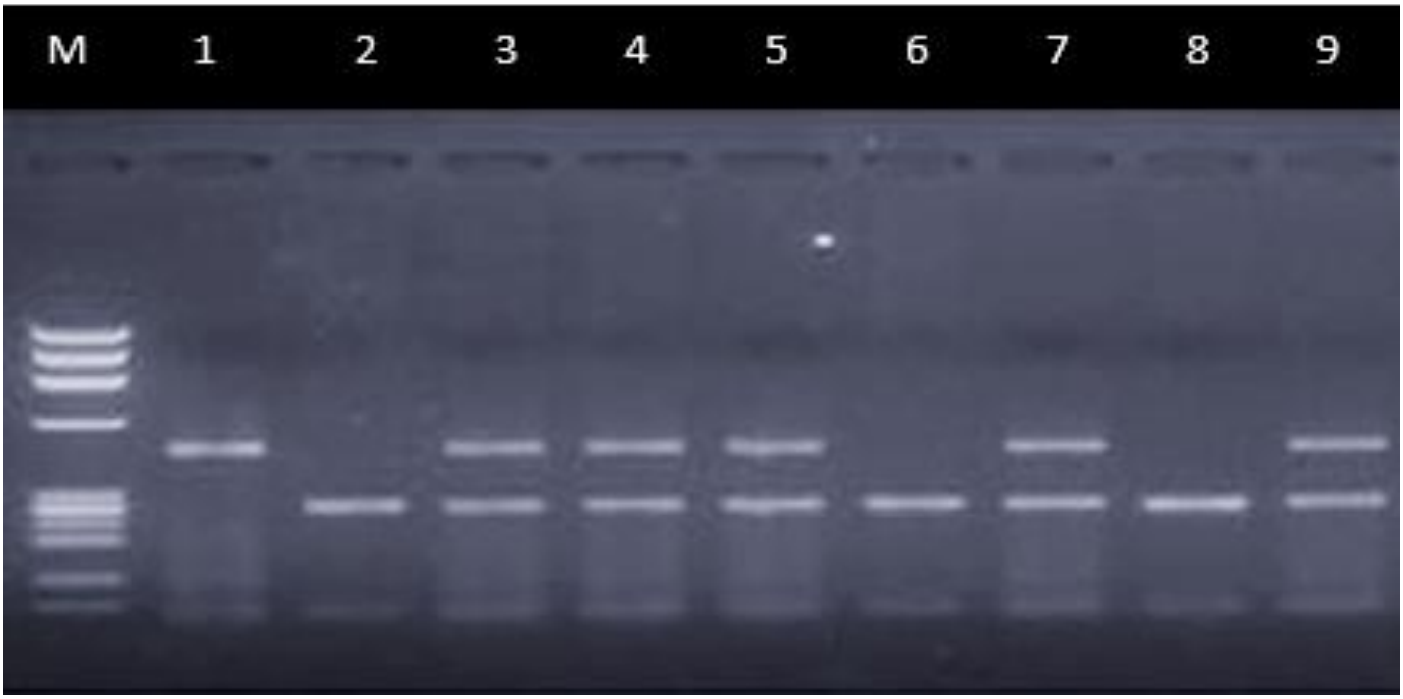

Figure-I: Determine the resistance pattern of MDR RPO B(Rifampicin) and Isoniazid for KAT G genes in gel electrophoresis.

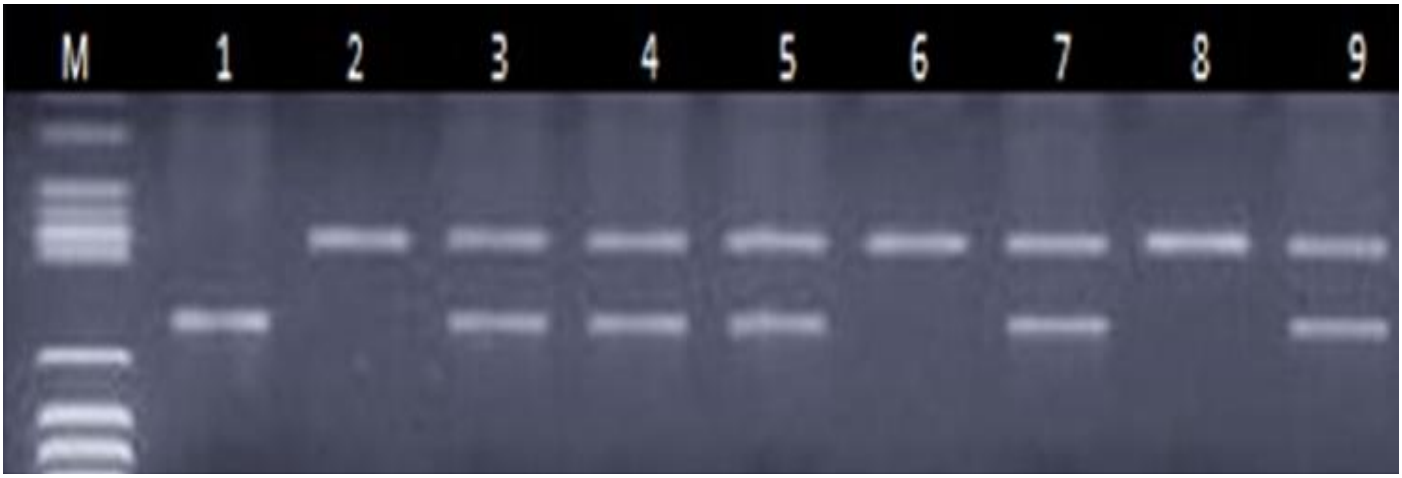

Figure-II: Determine the resistance pattern of XDR RPO B, KAT G, INH A, EMB A GENES IN gel electrophoresis.

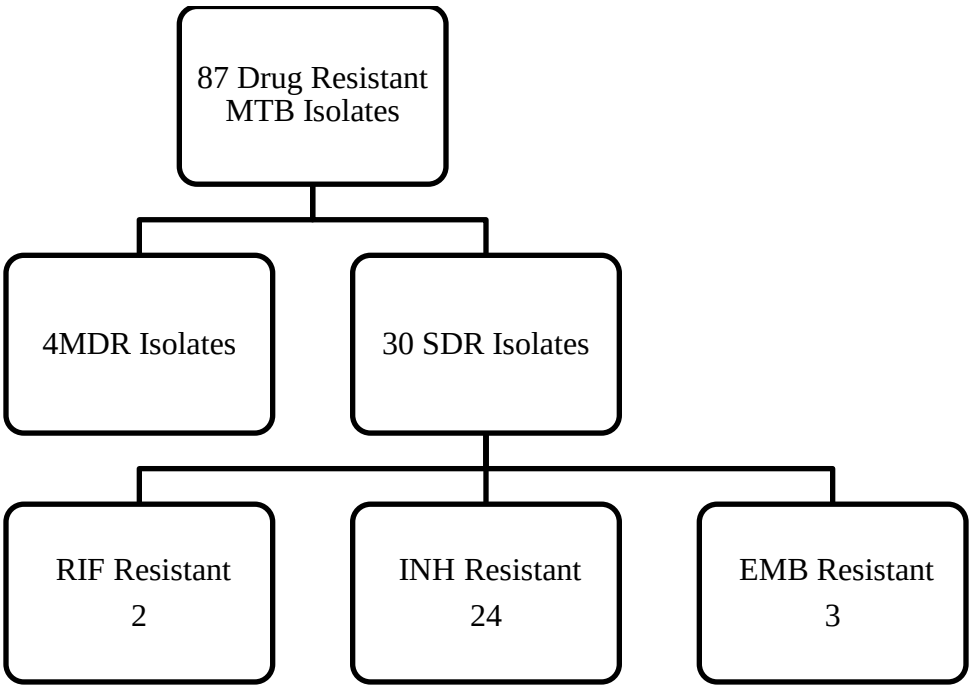

Figure-III: Quantity of culture isolates used in the retrospectively and subjected to phenotypic method.

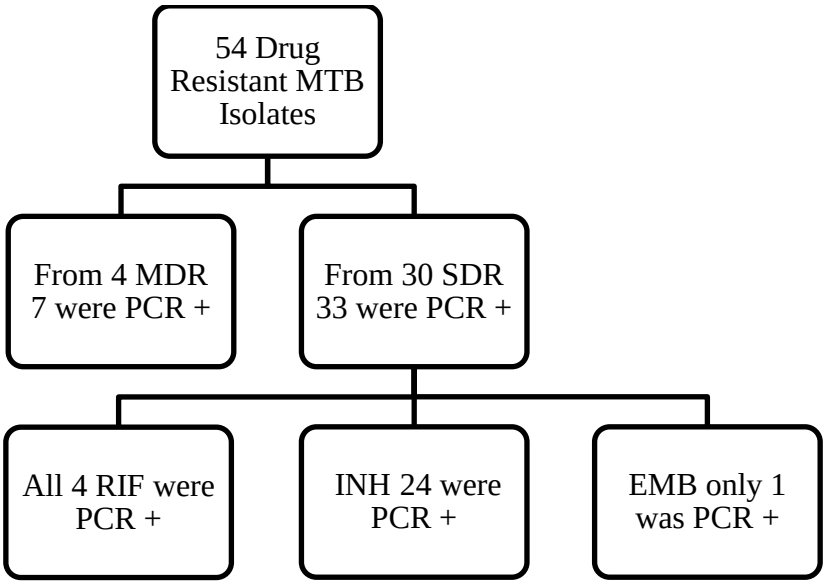

Figure-IV: Number of cultured isolates retrospectively selected for the study and analyzed by genotypic methods.

DISCUSSION

TB has been affecting humans for thousands of years. The culture-based diagnostic methods and drug susceptibility testing are still the gold standard for TB. These methods require at least 6–8 weeks for the outcomes. Conversely, molecular methods are promising for rapid and accurate diagnosis of drug-resistant TB. Most of the recently developed molecular methods are expensive, require a high degree of quality control, and are not available in the remote areas, where disease prevalence is high. Moreover, the WHO-approved Genxpert can diagnose resistance to RIF only. In Pakistan, the prevalence of drug-resistant TB is increasing, primarily due to the delayed diagnosis. An economical, easy molecular method is required to rapidly diagnose the drug-resistant TB in remote areas.

In the current study, we assessed phenotypic and geneotypic techniques that enabled the simultaneous identification of MTB resistance to RIF, INH, and EMB in 84 isolates of MTB-resistant bacteria from POF WAH CANTT, IDC, and Izzat Ali Shah. Primers were chosen for three loci that showed the highest frequency of RIF, INH, and EMB resistance-associated mutations for rpo B (531), kat G (316), and the emb B gene (345).

There are clear challenges with genotypic testing for INH resistance. First, INH resistance is linked to changes in at least three genes: kat G, inh A, and ahp C(2) Secondly, a change in kat G inside the regulatory areas of either ahp C or inhA.

Third, whereas certain mutations in kat G may not prov ide resistance to clinically relevant quantities of INH, o thers are linked to low-level resistance (deletion or S315T). Fourth, focusing on the codons that mutate most frequently globally may overlook strains that are representative of the local strain population or epidemic strains, as indicated by the relative frequency of a given mutation. Fifth, it's unclear how the overexpression of the ahpC gene and INH are related. The majority of INH-resistant isolates have been shown to carry mutations in kat G, inh A, or both. (11-13). A 305 bp portion of rpo B for RIF and a 458 bp section of the kat G gene for INH were employed in ou r study for MDR-TB. The MGIT 960 method was used to identify the 18 MDR isolates in total; of these isolates, 11 (17%) displayed distinctive banding patterns of concomitant rpo B and kat G mutations in multiplex PCR followed by gel electrophoresis. A comparable investigation on MDR MTB found a similar resistance trend in China.[14,15,16,17,18]

This study's primary goal was to determine how much time MTB geneotypic testing saves in comparison to phenotypic testing(3). When compared to MGIT culture or phenotypic test, genotypic testing resulted in a maximum time savings of 8 to 13 days (depending on the genotype). The fact that the study was conducted at a single site and that the sample size was manageable may be the most likely causes. This time-saving measure is critical because it enables doctors to start early medication and stop the spread of drug-resistant TB isolates (MDR or XDR).

Our study is limited in two ways. It was first done on specimens that tested positive for smears. Subsequently, only 21% of the isolates were multidrug resistant (MDR). In summary, our study's findings indicate that, when compared to MGIT culture and drug susceptibility testing, genotypic testing is a dependable technique with extremely excellent agreement. Significant time savings can aid in the start of early, successful therapy, particularly in MDR patients.

To determine its precise accuracy and dependability w hen used with INH, RIF, and other second-line anti-tuberculosis medications, further extensive research on a variety of medicines is required

CONCLUSION

The study's findings indicated that the genotypic testing employed in this investigation is a helpful method for quickly identifying drug-resistant tuberculosis in isolates and specimens, enabling the implementation of a first line of treatment. But the phenotypic approach could also be a helpful tool for precise patient management.

CONFLICT OF INTEREST

None

GRANT SUPPORT & FINANCIAL DISCLOSURE

Declared none

AUTHOR CONTRIBUTION

Saira Salim: Main idea and concept, final approval, accountable for all aspects of publication.

Syed Asim Ali Shah: Critical analysis, final approval, accountable for all aspects of publication.

Fareena Asim: Data collection, final approval, accountable for all aspects of publication.

Saba Anwar: Data interpretation, final approval, accountable for all aspects of publication.

Aniqa Shauqat: Review and methodology writing, final approval, accountable for all aspects of publication.

Naila Iqbal: Result and discussion writing, final approval, accountable for all aspects of publication.

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