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Research Article

Access The Diagnostic Accuracy of Genotypic Assays for The Rapid Detection of Drug-Resistant Mycobacterium Tuberculosis

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Abstract

An accurate diagnostic tool is crucial for detecting and effectively treating drug- resistant tuberculosis. This study aims to evaluate the performance of genotypic assays compared to the phenotypic Drug Susceptibility Test, which is considered the gold standard. In this study, 252 culture-positive samples were tested using the MTBDR*plus* and phenotypic liquid culture-based susceptibility testing. Additionally, 173 culture-positive samples were tested using MTBDR*plus* and Lowenstein-Jensen culture-based susceptibility testing. Furthermore, 93 culture-positive samples were tested using MTBDR*sl* and Liquid culture- based susceptibility testing. The agreement between GenoType MTBDR*plus* Ver-2.0 and BACTEC-MGIT-960 methods for isoniazid and rifampicin was perfect, with Kappa values of

0.75 (S.E.: 0.04) and 0.89 (S.E. 0.03), respectively. The test was statistically significant (p- < 0.00001). The agreement between GenoType MTBDR*plus* Ver-2.0 and Lowenstein-Jensen methods for the isoniazid and rifampicin drug was perfect (Kappa 0.84 with S.E.: 0.04 and Kappa 0.96 with S.E.: 0.02), and the test was statistically significant (p- < 0.00001). The overall agreement between GenoType MTBDR*sl* Ver-2.0 and BACTEC-MGIT-960 methods for all second-line drugs was good, except for Moxifloxacin, which showed statistically significant results ($p < 0.05$). While there was a considerable degree of agreement between the MTBDR*sl* and phenotypic Drug Susceptibility Test methods, the potential replacement of the phenotypic Drug Susceptibility Test by genotypic Drug Susceptibility Test for isoniazid and rifampicin is an enlightening finding. It can ensure timely and appropriate treatment in countries with a high burden of extremely drug-resistant tuberculosis. The tests showed excellent agreement, paving the way for a more efficient diagnostic process.

Keywords: Mycobacterium tuberculosis; Diagnostic Accuracy; Extremely Drug-Resistant; Multidrug-Resistant; GenoType MTBDRplus; Rifampicin-resistant

Introduction

Tuberculosis (TB) is a major public health issue and one of the leading global causes of death. Diagnosing TB is particularly challenging in low and middle-income countries with high disease rates. Rapid and timely diagnosis and treatment are essential for controlling TB and reducing its transmission [1]. However, the number of drug-resistant TB cases is increasing, making diagnosis and treatment difficult. Managing Drug-Resistant Tuberculosis (DR-TB) requires additional resources. Rapid and accurate diagnosis is crucial to identify all TB patients and start treatment promptly, which helps prevent transmission.

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Unfortunately, TB diagnostics have many limitations, including poor sensitivity, high complexity, and cost. Access to effective TB diagnostics remains a significant challenge.

The conventional drug susceptibility test for TB, endorsed by the WHO, is a phenotypic culture-based method. It involves exposing *M. tuberculosis* to specific concentrations of individual anti-TB drugs and detecting its growth. Although it is considered the gold standard, the conventional Drug Susceptibility Test takes a long time to produce results and poses a biosafety risk. In the past decade, major advances have been made in new diagnostic technologies for TB. Molecular drug susceptibility tests have been developed to check for mutations that confer resistance to specific anti-TB medications. The WHO-endorsed molecular technologies commonly used are the GenoType MTBDR*plus*, GenoType MTBDR*sl* (Hain Lifescience, Nehren, Germany), and GeneXpert MTB/RIF (Cepheid, Sunnyvale, CA, USA) tests. The advantages of these molecular drug susceptibility tests include faster turnaround time and less biohazard risk than the conventional Drug Susceptibility Test [2,3]. The MTBDR*plus* test is a line probe assay that simultaneously detects resistance to isoniazid and rifampicin drugs. This assay uses probes to detect mutations in specific regions of *katG* and *inhA* for isoniazid-resistant tuberculosis and *rpoB* for rifampicin-resistant tuberculosis. The MTBDR*sl* test is a line probe assay that can detect resistance to Fluoroquinolone and second- line injectable drugs. This MTBDRsl assay uses probes to mutations in specific regions of rrs and eis promoter genes to second-line injectable drugs and gyrA and gyrB genes to fluoroquinolones. Meanwhile, the GeneXpert MTB/RIF test detects essential first-line and second-line resistant *M. tuberculosis* drugs. The MTBDRplus test can detect multi-drug- resistant *M. tuberculosis* faster and safer than previous tests [4].

There is still a need for more reliable and efficient tests to detect tuberculosis and drug resistance quickly and accurately. Furthermore, better tests are required to confirm the absence of tuberculosis or identify individuals needing further testing. Increasing funding for tuberculosis research and development is essential to address these needs. This will accelerate the development, evaluation, and deployment of improved tests. This study aimed to assess the effectiveness of MTBDR*plus* and MTBDR*sl* assays in detecting Multidrug-Resistant (MDR) and Extensively Drug-Resistant (XDR) TB in patients. Multidrug-resistant is defined as resistance to at least isoniazid and rifampicin. In contrast, Extremely Drug-Resistant is defined as resistance to at least isoniazid, rifampicin with Fluoroquinolones, and any second-line injectable drugs.

Materials and Methods

Sample Collection and Transportation

The medical professionals have instructed drug-resistant TB patients to collect sputum samples in a pre-labelled, wide-mouth sterile container. After collecting the samples, patients should deliver them with a request form to the Intermediate Reference Laboratory facility. At each diagnostic site, the samples and forms are packed in a standard triple packaging container with an ice bag to maintain a temperature of 2-8°C. A registered courier is responsible for transporting all the samples to the Intermediate Reference Laboratory at the Government Hospital for Chest Diseases. Once they arrive at the laboratory, the samples are tested for culture, drug susceptibility, and molecular diagnostics. The Intermediate Reference Laboratory is certified by the Central TB Division in India to test the genotypic and phenotypic drug sensitivity of *M.tuberculosis.*

Sample Reception and Processing

The Intermediate Reference Laboratory at the Government Hospital for Chest Diseases received each sputum sample and the laboratory test request form. Upon receiving the samples, we checked to ensure that the laboratory request form was completed accurately, the Nikshay number was correct, the sample tube was correctly labelled, and there was no leakage. Once a sputum sample was accepted, a unique laboratory number was assigned for processing. The samples were then arranged in a clean rack and taken to Biosafety Level III facilities. All acceptable sputum samples were decontaminated using the N-acetyl-l-cysteine-sodium citrate- NaOH (NALC-NaOH) method. After centrifugation for 15 minutes at 3000g, the samples were decanted, and the pellets were re-suspended in 3 ml sterile phosphate buffer solution. Approximately 0.5 ml aliquots from each sample were used for microscopy, genotypic, and phenotypic drug susceptibility testing, and another 1 ml aliquot of each sample was stored at −80°C as a backup.

MGIT 960 Culture and Drug Susceptibility Testing (MGIT-DST)

The BACTEC-MGIT-960 test is used to detect the growth of M. tuberculosis in a drug- containing tube compared to a drug-free tube. The reliable BACTEC MGIT 960 instrument monitors the tubes for increased fluorescence and uses this analysis to determine susceptibility results. The instrument automatically interprets these results. The MGIT tubes in the BACTEC instrument are flagged by green light for no growth and red light for the growth of Mycobacterium on the front drawer. The culture growth in the MGIT tubes is confirmed for *M. tuberculosis* using Brain Heart Infusion agar (BHI), Ziehl-Neelsen stain, and MPT 64 antigen test before being subjected to Drug Susceptibility Testing (DST) against first-line drugs.

To begin the test, 800µl of BACTEC MGIT SIRE supplement was aseptically transferred to each of the five 7ml MGIT tubes, which were pre-labelled for each test isolate. It is crucial to maintain aseptic conditions during this step to ensure the accuracy of the test. Then, 100µL of the final concentration drug solution of S (1.0 µg/ml), I (0.1 µg/ml), R (1.0 µg/ml), and E (5.0 µg/ml) were aseptically transferred to all four pre-labelled drug-containing tubes. After that, 500µl of the 1:100 Growth Control suspension was aseptically transferred into the pre-labelled drug-free tube, and 500µl of working culture suspension was aseptically transferred into each of the four pre-labelled drug-containing tubes. The tubes were mixed thoroughly by gently inverting them three to four times after recapping them tightly. The order of the tubes in the AST carrier set was ensured after scanning, and then the tubes were loaded into BACTEC MGIT instruments. The BACTEC MGIT instrument completed the test, which interpreted the results as resistant or susceptible when the growth control tubes reached a growth unit of 400 or more. After scanning and printing, the tubes were removed from the instrument. Finally, the susceptibility testing was manually interpreted per sample as fully susceptible, mono-resistant, poly-resistant, or multidrug-resistant. A set of H37RV controls was included in all the runs to ensure the quality of the test.

Lowenstein-Jensen Culture and Drug Susceptibility Testing

Inoculum Preparation

Approximately 4 to 5 ml of sputum were collected and

transferred into a pre-labelled sterile centrifuge tube. Twice the sterile 4% NaOH solution volume was added to the sputum. The centrifuge tube was tightly capped and thoroughly mixed. Afterward, the tube was inverted to ensure the NaOH solution contacted all sides and inner portions of the caps. The centrifuge tube was incubated in an orbital shaker at 37°C for 15 minutes. Then, 15 ml of sterile distilled water was added to the centrifuge tube and mixed well. The mixture was then centrifuged at 3,000 x g for 15 minutes. The centrifuge tube was carefully removed from the centrifuge without shaking, and the supernatant fluid was slowly discarded into a 5% phenol solution container. The pellet was washed with sterile distilled water at 3,000 x g for 15 minutes, and the supernatant was decanted. Subsequently, the pellet was inoculated on two slopes of pre-labelled Lowenstein-Jensen medium slants using a sterile, cool 5 mm inoculation loop made from Nichrome wire. All the Lowenstein-Jensen media slopes were incubated at 37°C and checked for growth weekly for eight weeks.

Proportion Method

A McCartney bottle containing 1 ml of sterile distilled water and six 3mm glass beads was inoculated with approximately 4-5 mg of fresh culture. The contents were vortexed for 20- 30 seconds, and then 4-5 ml of sterile distilled water was slowly added while continuously shaking the bottle. The resulting mycobacterial suspension was carefully transferred to another pre-labelled clear and sterile McCartney bottle. The opacity of the bacterial suspension was adjusted by adding sterile distilled water to achieve a concentration of 1 mg/ml of tubercle bacilli, matching it with McFarland standard No.1. After preparing the bacterial inoculum, 100µl of it was inoculated on both drug-containing and drug-free Lowenstein-Jensen medium slants, with the drug-free slant serving as a control. The inoculated slants were incubated at 37°C for 21-28 days. Resistance was defined as growth on drug-containing tubes greater than 1% of the growth of the drug-free control medium for INH, RIF, EMB, and STR [8].

GenoType MTBDRplus Ver-2.0 assay for First-Line TB Drugs

The line probe assay (LPA) is a diagnostic method used to detect tuberculosis and identify Rifampicin (RIF) and Isoniazid (INH) resistance caused by mutations in *rpoB*, *inhA*, and *katG* genes. This method involves DNA extraction using the N-acetyl-L-Cysteine/NaOH method, multiplex PCR amplification, and reverse hybridization. To begin, about 1 mL of the liquid culture sample is transferred to a pre-labelled sterile 1.5 mL screw cap vial and then centrifuged for 15 minutes at 10,000× g. The resulting pellet is suspended in 100 µL of Lysis Buffer (A-LYS) and incubated at 95°C for 5minutes after discarding the supernatant. Approximately 100 µL of Neutralization Buffer (A-NB) is added, and the sample is briefly vortexed for 5sec. The vial is then centrifuged for 5 minutes at 10,000× g, and 40–80 µL of the supernatant is carefully transferred to a separate clean, sterile screw cap vial. The amplification mix (45 µL per PCR tube) is prepared in a DNA-free environment for the next step. The amplification Mixer A and B (AM-A and AM-B) contain all the necessary reagents for amplification. After thawing, AM-A and AM-B are mixed carefully. Then, 5 µL of DNA supernatant is added to corresponding PCR tubes, except for the contamination control, and 5 µL of water is added to one aliquot for the contamination control. Finally, all the PCR tubes are placed in the PCR instruments and run as per the manufacturer's instructions [9,10].

First, 20 µL of pre-warmed Denaturation Solution (DEN, blue) was dispensed into each well. Then, 20 µL of the amplified sample was added and incubated at room temperature for 5 minutes. After that, 1 mL of pre-warmed Hybridization Buffer (HYB, green) was carefully added to each well, and a strip was placed in each well of the GT Blot tray, ensuring that the solution fully covers the strips with the coated side facing upward. Next, they incubated the tray at 45°C for 30 minutes in the GT Blot instrument, shaking it frequently to mix the solution thoroughly. After incubation, aspirate the Hybridization Buffer using a sterile Pasteur pipette. 1 mL of stringent wash solution (STR, red) was added to each strip and incubated at 45°C for 15 minutes in the GT Blot instrument. The stringent wash solution was removed entirely using a separate Pasteur pipette. Each strip was rinsed once with 1 mL of Rinse Solution (RIN) for 1 minute in the GT Blot instrument. Then, 1 mL of diluted conjugate solution was added to each strip and incubated for 30 minutes in the GT Blot instrument. Removed the solution using a sterile Pasteur pipette and washed each strip twice for 1 minute with 1 mL of Rinse Solution (RIN) and once for 1 minute with approximately 1 mL of distilled water. 1 mL of diluted substrate solution was added to each strip, incubated for 3-20 minutes, and protected from direct light without shaking. The reaction was stopped when the bands became visible by briefly rinsing twice with distilled water. Finally, remove the strips from the tray using tweezers and paste them on an evaluation sheet provided in the kit [11,12].

Genotype MTBDRsl Ver 2.0 assay for Second-Line TB Drugs

Around 1 mL of culture suspension was transferred into a pre-labelled sterile 1.5 mL screw cap vial and then centrifuged at 10,000×g for 15 minutes. The supernatant was discarded, and the pellet was suspended in 100 µL of Lysis Buffer (A-LYS) and incubated at 95 °C for 5 minutes. Approximately 100 µL of Neutralization Buffer (A-NB) was added, and the sample was gently vortexed for 5 seconds. The liquid suspension was centrifuged for 5 minutes at 10,000×g, and 40–80 µL of the supernatant was carefully transferred to a clean, sterile microcentrifuge tube. The amplification mix (45 µL per PCR tube) was prepared in a room free from contaminating DNA. Amplification Mixers A and B (AM-A and AM-B) contain all the necessary reagents for amplification. After thawing, AM-A and AM-B were carefully mixed. Subsequently, 5 µL of DNA supernatant was added to corresponding PCR tubes, except for the contamination control, and 5 µL of water was added to one aliquot for the contamination control. All PCR tubes were placed in the PCR instruments, and the program was run as per the manufacturer's instructions [13].

To start the GT Blot procedure, 20 µL of Denaturation Solution (DEN, blue) was added to each well in the GT Blot tray, and then 20 µL of the amplified PCR product was added using a sterile pipette. The tray was then left at room temperature for 5 minutes. After that, 1 mL of pre-warmed Hybridization Buffer (HYB, green) was poured into each well, and the tray was gently shaken until the solution was well mixed. Each well had a pre-labeled strip placed into it using sterile tweezers. The tray was then placed in the GT-Blot instrument, maintaining a temperature of 45°C for 30 minutes. After incubation, the Hybridization Buffer was removed using a sterile Pasteur pipette. Subsequently, 1 mL of stringent wash solution (STR, red) was added to each strip and incubated at 45°C for 15 minutes in the GT-Blot instrument. The stringent wash solution was then entirely removed using a separate Pasteur pipette. Each strip was washed with 1 mL of Rinse Solution (RIN) for 1 minute in the GT-

Blot instrument. Following this, 1 mL of diluted conjugate was added to each strip-containing well and incubated for 30 minutes in the GT-Blot instrument. The solution was removed using a sterile Pasteur pipette, and each strip was washed twice for 1 minute with 1 mL of Rinse Solution (RIN) and once for 1 minute with approximately 1 mL of distilled water. Next, 1 mL of diluted substrate solution was added to each strip, incubated for 3-20 minutes, and protected from direct light without shaking. The reaction was stopped as soon as the bands became visible by briefly rinsing twice with distilled water. Finally, the strips were removed from the tray using tweezers and placed on an evaluation sheet provided in the kit [14].

Ethical Consideration

The study was approved by the Institutional Review Board of Indira Gandhi Government General Hospital and Postgraduate Institute (IRB No. GHIEC/2023-24/123) and was conducted in accordance with the principles of the declaration. Written informed consents were obtained. Prior to enrollment, each study participant received a standardized information sheet, and the study's objectives, risks, and benefits were explained to them. They were given the opportunity to ask questions, and those who agreed to participate signed an informed consent form. After enrollment, both groups with drug sensitivity and drug resistance were treated at a reputable medical facility based on the study findings. The samples were assigned unique study codes and were separated from the patient, with only age and sex being retained as socio-demographic information. It's important to note that the study samples did not impact the original patient results in any way.

Statistical Analysis

The study assessed the effectiveness of various genotypic and phenotypic tests by determining their specificity, sensitivity, Positive Predictive Value (PPV), Negative Predictive Value (NPV), and accuracy. We used MedCalc statistical [15] and Social Science software [16] for the analysis. The level of agreement between the tests was measured using Cohen's Kappa statistics, and tests with $P \leq 0.05$ were deemed statistically significant. The precision of the results was indicated by providing 95% confidence intervals (95% CI).

Results

Baseline Characteristics of The Study Population

One hundred seventy-three sputum samples were cultured using both Lowenstein- Jensen and the BACTEC-MGIT-960. These samples were also tested using the GenoType MTBDR*plus* Ver-2.0 assay to check for drug susceptibility in the treatment of tuberculosis. All 173 cultured specimens tested positive on both Lowenstein-Jensen and BACTEC-MGIT-960, and a drug susceptibility test was performed on both media. Out of the 173 sputum samples, genotypic and phenotypic drug susceptibility tests for first-line anti-TB drugs were validated, and 3 (1.7%) showed differences between the results of the genotypic and phenotypic tests. Within this group, 75 samples (43.4%) were diagnosed with rifampicin-resistant tuberculosis using the drug susceptibility tests, with 1 sample (0.6%) showing conflicting results for rifampicin resistance. The diagnostic accuracy of the MTBDR*plus* for the culture isolates is presented in Table 1. Among the 173 patients, the GenoType MTBDR*plus* Ver-2.0 assay demonstrated a high level of accuracy when compared with the phenotypic Lowenstein-Jensen DST. The sensitivity, specificity, positive and negative predictive values, and accuracy of the molecular assay for the isoniazid drug were 84.62%, 100.0%, 100.0%, 85.42%, and 91.91% respectively (Table 1). The respective values for rifampicin were 98.68%, 97.94%, 97.4%, 98.96%, and 98.27%. The overall agreement rates between GenoType MTBDR*plus* Ver-2.0 and Lowenstein-Jensen methods for the isoniazid and rifampicin drugs were perfect (Kappa 0.84 with S.E.: 0.04 and Kappa 0.96 with S.E.: 0.02). The statistical significance of these results ($p < 0.00001$) should still be confidence in the validity of the findings. When compared with the phenotypic (MGIT) Drug Susceptibility Test, the sensitivity, specificity, positive and negative predictive values, and accuracy of the molecular assay for the isoniazid drug were 84.62%, 100.0%, 100.0%, 85.42%, and 91.91% respectively (Table 3). The respective values for rifampicin were 98.68%, 97.94%, 97.4%, 98.96%, and 98.27%. The overall agreement rates between GenoType MTBDR*plus* Ver-2.0 and BACTEC-MGIT-960 methods for the isoniazid and rifampicin drugs were also excellent (Kappa 0.75 with S.E.: 0.04 and Kappa 0.89 with S.E.: 0.03), and the test was statistically significant ($p < 0.00001$).

In comparison to the MGIT Drug Susceptibility Test, the molecular assay for the isoniazid drug showed a sensitivity of 84.62%, specificity of 100.0%, positive predictive value of 100.0%, negative predictive value of 85.42%, and accuracy of 91.91%. For rifampicin, the sensitivity, specificity, positive and negative predictive values, and accuracy were 98.68%, 97.94%, 97.4%, 98.96%, and 98.27%, respectively. The agreement rates between GenoType MTBDR*plus* Ver-2.0 and BACTEC-MGIT-960 methods for these drugs were excellent, with a Kappa of 0.75 for isoniazid and 0.89 for rifampicin. The statistical significance for the test was $p < 0.00001$. In a study involving 173 patients, the comparison between two types of phenotypic drug susceptibility tests (L.J. Vs. MGIT) showed that the BACTEC-MGIT-960 test had 100.0% sensitivity, specificity, positive and negative predictive values, and accuracy for the isoniazid drug. Similar values for rifampicin were 100.0%, 98.98%, 98.68%, 100.0%, and 99.42%, respectively. The sensitivity, specificity, positive

Table 1: Diagnostic Performance of MTBDR plus for the detection of drug resistance in culture isolates compared to phenotypic culture-based (Lowenstein-Jensen method) susceptibility testing.

First-Line drug susceptibility test using Lowenstein-Jensen method (n=173)											
		INH		RIF		Drugs	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)	Accuracy (95% CI)
		R	S	R	S						
							84.62	100	100	85.42	91.91
FL	**R**	77	0	75	2	INH	(75.54 - 91.33)	(95.60 - 100.00)	(95.32 - 100.00)	(78.34 - 90.46)	(86.79 - 95.51)
LPA							98.68	97.94	97.4	98.96	98.27
	S	14	82	1	95	RIF	(92.89 - 99.97)	(92.75 - 99.75)	(90.49 - 99.33)	(93.13 - 99.85)	(95.02 - 99.64)
							100	100	100	100	100
FL	**R**	91	0	75	1	INH	(96.03 -100.00)	(95.60 - 100.00)	(96.03 -100.00)	(95.60 -100.00)	(97.89 - 100.00)
MGIT							100	98.98	98.68	100	99.42
	S	0	81	0	97	RIF	(95.20 -100.00)	(94.45 - 99.97)	(91.43 - 99.81)	(96.27 - 100.00)	(96.82- 99.99)

INH: Isoniazid; RIF: Rifampicin; LPA: Line Probe Assay; PPV: Positive Predictive Value; NPV: Negative Predictive Value.

Table 2: Frequency of genotypic-phenotypic (L-J) discrepancy to isoniazid or rifampicin drug resistant tuberculosis among 2 drug susceptibility tests (DSTs); MTBDRplus assay and phenotypic (LJ) drug susceptibility test.

GenoType MTBDR plus test	Phenotypic (L-J) DST (n=173)		K-Value (95% CI)	Standard error	p-Value (SL at p- < 0.05)
	Resistant	Susceptible			
Isoniazid					
Resistant	77(44.51%)	0	0.84	0.0407	
Susceptible	14(8.09%)	82(47.40%)	(0.76-0.92)		-
Rifampicin					
Resistant	75(43.35%)	2(1.16%	0.96 (0.92-	0.02012	< 0.00001
Susceptible	1(0.58%)	95(43.35%)	1.0)		

K: Kappa; SL: Significant level; L.J: Lowenstein-Jensen; MGIT: Microbial Growth Indicator Tube; DST: Drug Susceptibility Test

Table 3: Diagnostic Performance of MTBDRplus for the detection of drug resistance in culture isolates compared to phenotypic culture-based (BACTEC Mycobacterial Growth Indicator Tube -960 system) susceptibility testing.

First-Line drug susceptibility test using BACTEC Mycobacterial Growth Indicator Tube -960 system (n=252)											
		INH		RIF		Drugs	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)	Accuracy (95% CI)
		R	S	R	S						
							79.70	95.80	95.50	80.85	87.30
FL	**R**	106	5	101	8	INH	(71.86 - 86.17)	(90.47 - 98.62)	(89.95 - 98.05)	(75.05 - 85.56)	(82.55 - 91.15)
LPA							94.39	94.37	92.66	95.71	94.38
	S	27	114	6	137	RIF	(88.19 - 97.91)	(89.20 - 97.54)	(86.54 - 96.12)	(91.11 - 97.99)	(90.75 - 96.89)

INH: Isoniazid; RIF: Rifampicin; FL LPA: First-line Line Probe Assay; PPV: Positive Predictive Value; NPV: Negative Predictive Value.

Table 4: Frequency of genotypic-phenotypic (MGIT) discrepancy to isoniazid or rifampicin drug resistant tuberculosis among 2 drug susceptibility tests (DSTs); MTBDR plus assay and phenotypic (MGIT) drug susceptibility test.

GenoType MTBDRplus test	Phenotypic (MGIT)DST (n=252)		K-Value (95% CI)	Standard error	p-Value (SL at p- <0 .05)
	Resistant	Susceptible			
Isoniazid					
Resistant	106(42.06%)	5(1.98%)	0.75	0.04105	<0.00001
Susceptible	27(10.71%)	114(45.24%)	(0.67-0.83)		
Rifampicin					
Resistant	101(40.08%)	8(3.17%)	0.89	0.02945	<0.00001
Susceptible	6(2.38%)	137(54.37%	(0.83-0.94)		

K: Kappa; SL: Significant level; L.J: Lowenstein-Jensen; MGIT: Microbial Growth Indicator Tube; DST: Drug Susceptibility Test

and negative predictive values, and accuracy for streptomycin were 90.70%, 98.89%, 98.73%, 91.75%, and 94.89%, respectively. The MGIT test showed 89.66% sensitivity, 99.14% specificity, 98.11% positive predictive value, 95.04% negative predictive value, and 95.98% accuracy for ethambutol. The overall agreement rates between BACTEC-MGIT-960 and Lowenstein-Jensen methods for these drugs were perfect, and the test was statistically significant with $p < 0.00001$.

Of 252 sputum samples tested for drug susceptibility to first-line anti-TB drugs using genotypic and phenotypic (BACTEC-MGIT-960) methods, 14 samples (5.6%) showed conflicting results. Among these samples, 101 (40.1%) were identified as having rifampicin- resistant tuberculosis by one of the tests, and 6 (2.4%) had inconsistent results for rifampicin resistance. The molecular assay for isoniazid drug sensitivity showed a sensitivity of 79.70%, specificity of 95.80%, positive predictive value of 95.05%, negative predictive value of 80.85%, and accuracy of 87.30% compared to the phenotypic (MGIT) drug susceptibility test. For rifampicin, the respective values were 94.39%, 94.37%, 92.66%, 95.71%, and 94.38%. The GenoType MTBDR*plus* Ver-2.0 and BACTEC-MGIT-960 methods showed perfect overall agreement rates for isoniazid and rifampicin, with Kappa values of 0.75 (S.E.: 0.04) and 0.89 (S.E.: 0.03), respectively. A p-value of less than 0.00001 represented the statistical significance of the test.

The GenoType MTBDR*plus* Ver-2.0 and BACTEC-MGIT-960 methods showed perfect overall agreement rates for isoniazid and rifampicin, with Kappa values of 0.75 (S.E.: 0.04) and 0.89 (S.E.: 0.03), respectively. A p-value of less than 0.00001 represented the statistical significance of the test. The GenoType MTBDR*plus* Ver-2.0 and BACTEC-MGIT- 960 methods had high overall agreement rates for diagnosing resistance to isoniazid and rifampicin, with Kappa values of 0.75 (S.E.: 0.04) and 0.89 (S.E.: 0.03), respectively. The p- value of less than 0.00001 indicated the statistical significance of the test. A total of 93 sputum samples were tested for genotypic and phenotypic drug susceptibility for second-line anti-TB drugs using the BACTEC-MGIT-960 method. The molecular assay for Levofloxacin showed a sensitivity of 74.29%, specificity of 74.14%, positive predictive value of 63.41%, negative predictive value of 82.69%, and accuracy of 74.19%. Moxifloxacin's respective values were 58.33%, 60.87%, 34.15%, 80.77%, and 60.22%. For Capreomycin, the values were 50.00%, 85.71%, 62.50%, 78.26%, and 74.19%. The assay for Kanamycin/Amikacin showed a sensitivity of 68.00%, specificity of 89.71%, positive predictive value of 70.83%, negative predictive value of 88.41%, and accuracy of 83.87%.

Table 7 clearly illustrates the diagnostic accuracy of MTBDR*sl* for the culture isolates. The GenoType MTBDR*sl* method demonstrated a high percentage of resistance detection for Levofloxacin, Moxifloxacin, Kanamycin, Capreomycin, and Amikacin, with values of 44.1% (41/93), 44.1% (41/93), 25.05% (24/93), 25.05% (24/93), and 25.05% (24/93), respectively.

The BACTEC MGIT-960 system also showed a significant percentage of resistance detection for these drugs, with values of 37.6% (35/93), 25.8% (24/93), 32.3% (30/93), 26.9% (24/93), and 26.9% (24/93), respectively. For Levofloxacin, the BACTEC-MGIT-960 method had a moderate agreement (Kappa 0.47 with S.E.: 0.09, $p < 0.05$). Moxifloxacin showed poor agreement (Kappa 0.16 with S.E.: 0.10, $p > 0.5$), Capreomycin had fair agreement (Kappa 0.47 with S.E.: 0.10, $p < 0.05$), Kanamycin had moderate

Table 5: Diagnostic Performance of BACTEC MGIT-960 for the detection of drug resistance in culture isolates compared to phenotypic culture-based (Lowenstein- Jensen method) susceptibility testing.

First-Line drug susceptibility test using Lowenstein-Jensen method (n=173)															
		STR		INH		RIF		EMB		Drugs	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)	Accuracy
		R	S	R	S	R	S	R	S						
FL											90.7	98.89	98.73	91.75	94.89
STR											(82.49 - 95.90)	(93.96 - 99.97)	(91.73 - 99.82)	(85.18 - 95.56)	(90.51 - 97.64)
	R	75	1	91	0	75	1	52	1						
											100	100	100	100	100
INH											(96.03 - 100.00)	(95.60 -100.00)	(96.03 - 100.00)	(95.60 - 100.00)	(97.89 - 100.00)
MGIT											100	98.98	98.68	100	99.42
RIF											(95.20 - 100.00)	(94.45 - 99.97)	(91.43 - 99.81)	(96.27 - 100.00)	(96.82 - 99.99)
	S	8	89	0	82	0	97	6	114						
											89.66	99.14	98.11	95.04	95.98
EMB											(78.83 - 96.11)	(95.29 - 99.98)	(88.06 - 99.73)	(89.98 - 97.61)	(91.89 - 98.37)

INH: Isoniazid; RIF: Rifampicin; LPA: Line Probe Assay, PPV: Positive Predictive Value; NPV: Negative Predictive Value

Table 6: Frequency of phenotypic (MGIT)-phenotypic (L.J) discrepancy to first-line drug resistant tuberculosis among 2 types drug susceptibility tests.

Phenotypic (MGIT)DST	Phenotypic (L.J) DST (n=173) Resistant		K-Value	Standard error	p-Value (SL at p- < 0.05)
	Resistant	Susceptible			
Streptomycin					
Resistant	75(43.35%)	1 (0.58%)	0.9	0.03382	<0.00001
Susceptible	8(4.62%)	89(51.45%)	(0.83-0.96)		
Isoniazid					
Resistant	91(52.6%)	0	1	0	-
Susceptible	0	82(47.4%)	(1.0-1.0)		
Rifampicin					
Resistant	75(43.35%)	1(0.58%)	0.99	0.01172	-
Susceptible	0	97(56.07%)	(0.97-1.0)		
Ethambutol					
Resistant	52(30.06%)	1(0.58%)	0.9	0.03426	<0.00001
Susceptible	6(3.47%)	114(65.90%)	(0.84-0.97)		

K: Kappa; SL: Significant level; L.J: Lowenstein-Jensen; MGIT: Microbial Growth Indicator Tube; DST: Drug Susceptibility Test.

Table 7: Diagnostic accuracy of MTBDRsl for the detection of drug resistance in culture isolates compared to phenotypic culture-based (BACTEC Mycobacterial Growth Indicator Tube -960) susceptibility testing.

Second-Line drug susceptibility test using BACTEC Mycobacterial Growth Indicator Tube -960 system (n=93) LEV															
	LEV		MOX		CAP		KAN		AMK		Drugs	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)	Accuracy (95% CI)
	R	S	R	S	R	S	R	S	R	S						
												74.29	74.14	63.41	82.69	74.19
											LEV	(56.74 - 87.51)	(60.96 - 84.74)	(51.82 - 73.64)	(72.73 - 89.54)	(64.08 - 82.71)
R	26	15	14	27	15	9	17	7	17	7						
												58.33	60.87	34.15	80.77	60.22
											MOX	(36.64 - 77.89)	(48.37 - 72.40)	(24.88 - 44.81)	(71.61 - 87.49)	(49.54 - 70.22)
												50	85.71	62.5	78.26	74.19
											CAP	(31.30 - 68.70)	(74.61 - 93.25)	(45.22 -77.09)	(71.28 - 83.93)	(64.08 - 82.71)
												68	89.71	70.83	88.41	83.87
S	9	43	10	42	15	54	8	61	8	61						
											KAN	(46.50 - 85.05)	(79.93 - 95.76)	(53.39 - 83.74)	(81.07 - 93.14)	(74.80 - 90.68)
												68	89.71	70.83	88.41	83.87
											AMK	(46.50 - 85.05)	(79.93 - 95.76)	(53.39 - 83.74)	(81.07 - 93.14)	(74.80 - 90.68)

LEV: Levofloxacin; MOX: Moxifloxacin; CAP: Capreomycin; KAN: Kanamycin; AMK: Amaikacin; SL LPA: Second-line Line Probe Assay; PPV: Positive Predictive Value; NPV: Negative Predictive Value; FQ: Fluroquinolone; SLIJ: Second-line Injectable.

agreement (Kappa 0.58 with S.E.: 0.10, p< 0.05), and Amikacin had moderate agreement (Kappa 0.58 with S.E.: 0.10, p < 0.05). All second-line drugs, except Moxifloxacin, showed statistically significant results (p < 0.05), as indicated in Table 8. A total of 93 sputum samples were tested for genotypic and phenotypic drug susceptibility for second-line anti-TB drugs using the BACTEC-MGIT-960 method. The molecular assay for Levofloxacin showed a sensitivity of 74.29%, specificity of 74.14%, positive predictive value of 63.41%, negative predictive value of 82.69%, and accuracy of 74.19%. Moxifloxacin's respective values were 58.33%, 60.87%, 34.15%, 80.77%, and 60.22%. The respective values for Capreomycin were 50.00%, 85.71%, 62.50%, 78.26%, and 74.19%. The molecular assay for Kanamycin/Amaikacin showed a sensitivity of 68.00%, specificity of 89.71%, positive predictive value of 70.83%, negative predictive value of 88.41%, and accuracy of 83.87%. Table 7 shows the diagnostic accuracy

Table 8: Frequency of genotypic-phenotypic (MGIT) discrepancy to Second line drug resistant tuberculosis among 2 drug susceptibility tests (DSTs); MTBDRsl assay and phenotypic (MGIT) drug susceptibility test

GenoType MTBDRsl test	Phenotypic (MGIT) DST (n=93)		K-Value	Standard error	p-Value (SL at p- < 0.05)
	Resistant	Susceptible			
Levofloxacin					
Resistant	26(27.95%)	15(16.13%)	0.36	0.09216	< 0.00001
Susceptible	9(9.68%)	43(46.24%)	(0.15-0.56)		
Moxifloxacin					
Resistant	14(15.05%)	27(29.03)	0.17	0.09607	0.163487
Susceptible	10(10.75%)	42(45.16%)	(0.03-0.34)		
Capreomycin					
Resistant	15(16.13%)	9(9.68%)	0.38	0.10344	0.000613
Susceptible	15(16.13%)	54(58.04%)	(0.14-0.58)		
Kanamycin					
Resistant	17(18.28%)	7(7.53%)	0.58	0.09594	< 0.00001
Susceptible	8(8.60%)	61(65.59%)	(0.40-0.77)		
Amaikacin					
Resistant	17(18.28%)	7(7.53%)	0.58	0.09594	< 0.00001
Susceptible	8(8.60%)	61(65.59%)	(0.40-0.77)		

K: Kappa; SL: Significant level; L.J: Lowenstein-Jensen; MGIT: Microbial Growth Indicator Tube; DST: Drug Susceptibility Test.

of GenoType MTBDR*sl* for the culture isolates. The percentage of resistance detection by GenoType MTBDR*sl* for Levofloxacin, Moxifloxacin, Kanamycin, Capreomycin, and Amaikacin were 44.1% (41/93), 44.1% (41/93), 25.05% (24/93), 25.05% (24/93), and 25.05% (24/93), respectively. The percentage of resistance detection by the BACTEC MGIT-960 system for Levofloxacin, Moxifloxacin, Kanamycin, Capreomycin, and Amaikacin were 37.6% (35/93), 25.8% (24/93), 32.3% (30/93), 26.9% (24/93), and 26.9% (24/93), respectively. The BACTEC-MGIT-960 method for Levofloxacin had a moderate agreement (Kappa 0.47 with S.E.: 0.09, p< 0.05). Moxifloxacin had poor agreement (Kappa 0.16 with S.E.: 0.10, p > 0.5), Capreomycin had fair agreement (Kappa 0.47 with S.E.: 0.10, p<0.05), Kanamycin had moderate agreement (Kappa 0.58 with S.E.: 0.10, p < 0.05), and Amaikacin had moderate agreement (Kappa 0.58 with S.E.: 0.10, p < 0.05). All second-line drugs, except Moxifloxacin, showed statistically significant results (p < 0.05) (Table 8).

Discussions

Drug-resistant Tuberculosis (TB) is a significant threat to TB control programs worldwide, particularly in low- and middle-income countries. Misuse of anti-TB drugs increases the risk of drug resistance, making it challenging to prevent and treat TB effectively. Timely and accurate diagnosis of drug-resistant TB is crucial to identify all infected patients and start treatment promptly, leading to successful outcomes and preventing further transmission. However, current diagnostic tools have limitations such as low sensitivity, high costs, and complexity, which hinder access to effective TB diagnostics. In developing and high- burden countries, molecular tests like MTBDR*plus* and MTBDR*sl* are increasingly used to diagnose Multi-Drug-Resistant Tuberculosis and Extremely Drug-Resistant Tuberculosis. At the same time, conventional culture-based DST is considered the gold standard for drug resistance testing of *M. tuberculosis*.

The World Health Organization (WHO) states that determining the most effective tuberculosis treatment regimen relies on accurate susceptibility testing of M. tuberculosis to anti-TB drugs. However, the precision of susceptibility testing results varies depending on the drug being tested and the testing method used. In a recent study, we compared the performance of the MTBDR*plus* ver.2.0 to the Lowenstein-Jensen method for detecting susceptibility to first-line drugs. The MTBDR*plus* assay showed a sensitivity of 84.62% and specificity of 100% for detecting susceptibility to isoniazid (INH) and a sensitivity of 98.68% and specificity of 97.94% for detecting susceptibility to Rifampicin (RIF). However, a study by Rahman et al. in 2022 [17] reported different results. They found that the sensitivity and specificity for detecting rifampicin resistance using the MGIT Drug Susceptibility Test were 88.7% and 97.8%, and for isoniazid, were 88% and 97.4%, respectively. It's important to note that mutations in the *inhA* and *katG* genes cause most isoniazid resistance. However, mutations in other genetic regions, such as *ahpC*, *fabG*1, and *ndh* genes, have also been associated with resistance. Unfortunately, the MTBDRplus ver.2.0 assay cannot detect these genes. Our study found that the MTBDR*plus* ver.2.0 assay had a higher sensitivity for detecting rifampicin resistance but lower sensitivity for detecting isoniazid resistance compared to the study by Rahman et al. in 2022 [17]. This suggests that mutations causing isoniazid resistance, apart from the typical *katG*315 and *inhA* promoter region mutations, are more common in highly resistant tuberculosis cases [18].

Our research compared the MTBDR*plus* ver.2.0 with the BACTEC MGIT 960 system to determine their effectiveness in detecting drug susceptibility to first and second-line drugs. The sensitivity and specificity of the MTBDR*plus* assay for isoniazid were 79.7% and 95.8%, respectively. For rifampicin, the sensitivity and specificity were 94.39% and 94.37%, respectively. In a separate study, Yigzaw et al. [13] reported the sensitivity and specificity for rifampicin resistance using the MGIT Drug Susceptibility Test as 75% and 100%, respectively, and for isoniazid as 94.4% and 100%, respectively. Meanwhile, Rahman et al. [17] reported the sensitivity and specificity for rifampicin resistance using the MGIT Drug Susceptibility Test as 90% and 94.5%, respectively, and for isoniazid as 97.6% and 89.9%, respectively. Our study revealed a higher sensitivity for rifampicin resistance but a lower sensitivity for isoniazid compared to the studies reported by Yigzaw et al. [13] and Rahman et al. [17].

Our study evaluated the performance of the BACTEC MGIT 960 system compared to the Lowenstein-Jensen method for detecting drug susceptibility to four first-line drugs (excluding Pyrazinamide). We discovered that the BACTEC MGIT 960 system demonstrated sensitivity and specificity of 90.70% and 98.89% for detecting Streptomycin resistance, 100% and 100%

for Isoniazid, 100% and 98.98% for Rifampicin, and 89.66% and 99.14% for Ethambutol, respectively. In contrast, Rahman et al. [17] reported sensitivity and specificity for detecting Streptomycin resistance against the Lowenstein-Jensen method at 61.3% and 91.3%, for Isoniazid at 97.6% and 89.9%, for Rifampicin at 90.0% and 94.5%, and for Ethambutol at 44.9% and 92.2%, respectively. Our study revealed higher sensitivity and specificity for all four first-line TB drugs (except Pyrazinamide) compared to the previous study by Rahman et al [17].

In our study, we compared the effectiveness of the MTBDR*sl* ver: 2.0 (Line Probe Assay) and the BACTEC-MGIT-960 system in detecting drug susceptibility to second-line drugs. Our findings revealed that the MTBDR*sl* ver.2.0 had moderate sensitivity and specificity for detecting resistance to Levofloxacin, with rates of 74.29% and 74.14%, respectively. The rates for detecting resistance to Moxyfloxacin were 58.33% and 60.87%, while for Capreomycin, the MTBDR*sl* ver.2.0 had 50% and specificity rates of 85.71%. Additionally, the rates for Kanamycin and Amikacin were 68% and 89.71% for both, respectively. In contrast, Bouzouita et al. reported much higher sensitivity and specificity rates for the GenoType MTBDR*sl* assay against the MGIT Drug Susceptibility Test. Specifically, for Levofloxacin resistance, the rates were 92.8% and 100%; for Kanamycin, they were 100% and 100%; for Capreomycin, they were 75% and 100%; and for Amikacin, they were 100% and 100%, respectively.

Our study found that the sensitivity rates of the Genotype MTBDR*sl* assay were generally lower than those reported by Bouzouita et al. in 2021. The mutation at low MIC (≤ 0.5 µg/mL) could be the main reason for higher discordance between the genotypic and phenotypic assay. There may be inconsistencies between phenotypic drug susceptibility tests and current rapid genotypic assays, possibly because not all mutations that cause resistance to anti-TB drugs are included in rapid genotypic assays. Some resistant mutations can result in a variable phenotypic expression of drug resistance, which can be low, moderate, or high. Notably, silent mutations can occur at the genetic level without changing drug susceptibility patterns. Also, neutral mutations may result in LPA detecting more resistant isolates than the phenotypic MGIT 960. On the other hand, MGIT 960 may fail to detect low-level resistance mutations below the drug breakpoint, which can have evolutionary consequences.

A recent Indian study by Radhakrishnan et al. showed the sensitivity and specificity rates for the GenoType MTBDR*sl* assay for Kanamycin as 76% and 89% and for Capreomycin as 47% and 94%, respectively. Detecting mutations in *gyrA* and *gyrB* genes can help predict the presence and level of moxifloxacin resistance. Mutations within the Quinolone Resistance-Determining Region (QRDR) of *gyrA* account for 42 to 100% of fluoroquinolone resistance in *M. tuberculosis,* with codons 90, 91, and 94 being the most mutated sites. Different mutations in *gyrA* are associated with varying levels of Moxifloxacin resistance. Kambly et al. reported that strains with mutations of Ala90Val or Ser91Pro had an MIC of 1.0 µg/mL for moxifloxacin, whereas isolates with mutations at Asp94Ala, Asp94Asn/Tyr, Asp94Gly, and Asp94His were associated with an MIC of 2.5 µg/mL. Our research shows that the GenoType MTBDR*plus* ver.2.0 can effectively identify resistance to isoniazid and rifampicin, mainly when unavailable phenotypic drug susceptibility testing. This method has high sensitivity and specificity with minimal discrepancies, making it useful for promptly determining a patient's MDR status. Compared to the MGIT 960 system, the GenoType MTBDR*plus* is an accurate and efficient way to detect drug-resistant TB [23]. However, it is essential to establish appropriate laboratory design, standard biosafety procedures, and quality control measures to prevent contamination and reduce costs, especially in resource-limited settings. While the MGIT 960 system is less expensive than the GenoType MTBDR*plus*, it is more complex and necessitates strict biosafety standards.

Conclusion

Based on our research, the GenoType MTBDR*plus*Ver.2.0 test can accurately detect resistance to isoniazid, rifampicin, and multidrug-resistant tuberculosis. It offers a reliable alternative to traditional resistance detection methods for isoniazid and rifampicin. The test is precious in identifying low-level isoniazid resistance (*inhA*), allowing healthcare providers to adjust isoniazid dosage to improve its effectiveness and avoid using ethionamide due to cross- resistance. Conversely, if the test identifies high-level isoniazid resistance (*katG*), it suggests that isoniazid may not be a suitable treatment option. The GenoType tests can provide valuable information on *M. tuberculosis* resistance patterns within 2-3 days. However, it's important to note that the sensitivity of the GenoType MTBDR*sl* V2.0 for detecting resistance to certain drugs is still debatable, with reported ranges of 57–100% for some drugs and 25–100% for others. There is increasing recognition of discrepancies between genetic and phenotypic test results, particularly in regions with high tuberculosis incidence, such as India. These discrepancies may not solely stem from the absence of all drug-resistance mutations in the genetic assays but could also involve the presence of neutral or silent mutations. Another potential factor contributing to these differences is the possibility that the MGIT 960 test may not be able to detect low-level resistance mutations falling below the drug breakpoint, which could have significant evolutionary implications. Additionally, the test does not include additional probes for the *tlyA* gene. Despite these discrepancies, we recommend refining the GenoType assay to enhance its sensitivity and effectiveness in clinical applications.

Author Statements

Conflicts of Interest

The authors declare no conflict of interest.

Author Contributions

Muthukumar Anbalazhi, Pradhabane Gunavathy, Brammacharry Usharani: Conceptualization, Methodology Investigation and Writing Original Draft, Data curation, Formal Analysis, Writing Review and Editing; Ramachandra Venkateswari: Data curation and Formal Analysis; Balasundaram Revathi: Validation, Writing Review and Editing; Muthaiah Muthuraj: Conceptualization, Supervision, Project Administration, Validation, Writing Review and Editing.

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