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Original Article

Synergy of Xpert (MTB/RIF) and Line probe assay for detection of rifampicin resistant strains of *Mycobacterium tuberculosis*

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Abstract

Introduction: Early diagnosis and successful treatment of drug-resistant tuberculosis (TB) demands rapid, precise, and consistent diagnostic methods to minimise the development of resistance. Therefore, this comparative study was designed to evaluate the diagnostic performance of Xpert (MTB/RIF) and Line probe assay (LPA) for detecting drug-resistant TB.

Methodology: This study comprised 389 (279 pulmonary and 110 extrapulmonary) samples from patients suspected of having TB. All samples were subjected to Xpert (MTB/RIF), LPA, solid culture, and drug-susceptibility testing. Out of 320 samples, only 180 culture (gold standard) positive were included in the final evaluation. The diagnostic characteristics for methods used were determined by calculating diagnostic sensitivity, specificity, and predictive values. The agreement between all methods was determined by calculating the kappa coefficient.

Results: The sensitivity and specificity for Xpert (MTB/RIF) for detecting TB were 88.5% and 96.4%, respectively, against the solid culture. On the other hand, LPA showed sensitivity and specificity at 94.3% and 100%, respectively. Xpert (MTB/RIF) showed moderate agreement (kappa 0.65, $p < 0.01$) – (73.3% sensitivity; 97.6% specificity) for the detection of rifampicin resistance. However, LPA achieved better diagnostic accuracy (kappa 0.80, $p < 0.01$) – (84.6% sensitivity; 98.4% specificity) against drug-resistant TB.

Conclusions: Xpert (MTB/RIF) and LPA have outstanding diagnostic sensitivity and specificity against RIF resistance with a shorter turnaround time, which could result in a substantial therapeutic outcome. Our findings showed LPA superiority over Xpert (MTB/RIF) for drug resistance. However, due to operational challenges, the requirement of technical expertise and infrastructure issues, LPA cannot be used as point-of-care testing in resource-limited countries.

Key words: Multidrug resistant tuberculosis (MDR-TB); Line probe assay (LPA); Xpert (MTB/RIF); drug susceptibility testing (DST).

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Introduction

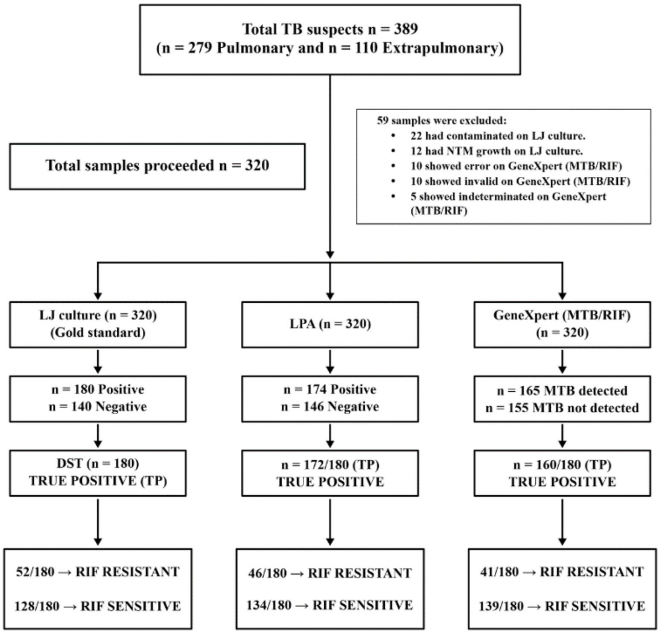
Multidrug-resistant tuberculosis (MDR-TB) has been considered a global health challenge in recent years, especially in countries with an emerging trend of MDR-TB cases [1]. To halt the spread of uncontrolled infectious bacteria of MDR-TB by prompt diagnosis and initiation of anti-TB drugs plays a pivotal role in resource-limited countries [2]. In 2020, the World Health Organization (WHO) reported that more than half a million people worldwide were infected with MDR-TB, with only 156,071 MDR-TB cases being treated [3,4]. Pakistan was ranked sixth among TB-incident countries, with a population of 210 million people, where approximately 1.5 million people suffer from TB [5]. Pakistan ranked fifth, with around 15,000/518,000 MDR-TB cases in the 30 high TB burden countries (HBC) [6]. This significant emergence of MDR-TB has been directly associated with the gap in diagnosis, improper usage of the imperfect and inadequate panel of drugs, inconsistency in treatment follow-up, and unavailability of social support programs [7]. Consequently, it is necessary for the Pakistani TB control program (NTP) to adopt practical disease-control strategies and to intensify awareness and management practices for early detection in conjunction with stringent infection-control programs [8]. Therefore, more efficient detection methods for MDR-TB and awareness strategies should be followed and implemented per WHO recommendations.

Traditionally, phenotypic drug-susceptibility testing (DST) on Löwenstein–Jensen medium (LJ) or *Mycobacterium* growth indicator tube (MGIT) are the diagnostic methods used for detecting MDR-TB. Though these diagnostic tools are considered the "gold standard" for MDR-TB, high contamination rates constrain them, requiring dedicated laboratories, skilled

personnel, and lengthy turnaround time (over 84 days for LJ culture and 42 days for DST). These shortcomings impede their extensive execution in our healthcare system [9,10]. Therefore, implementing more sensitive and rapid diagnostic technologies with improved detection times may significantly improve the management of MDR-TB. The two-fold challenge of low indicative sensitivity of microscopy and the technological challenge of executing a TB culture (DST) poses a difficulty in detecting resistance in *Mycobacterium tuberculosis* (*MTB*) and initiating TB second-line treatment. Hence, there is a need for a user-friendly, easily accessible, rapid, and reliable assay to help manage MDR-TB in Pakistan.

In developing countries, the WHO has approved more rapid molecular Xpert (MTB/RIF) technology and LPA (also known as MTB-DR-plus) for detecting drug resistance in *MTB* strains [11]. Xpert (MTB/RIF) is a quantitative real-time polymerase chain reaction (PCR) technology, while LPA is a molecular assay based on PCR and hybridization, which detects resistance in the rifampicin (RIF) and isoniazid (INH) regions of the *MTB* complex within 1 day [11,12]. Nevertheless, in countries with emerging trends of MDR-TB cases, it is vital to use LPA to study the strain diversity and possibility of new mutations. The attractive part of these technologies is their rapid detection time of just 2 hours for Xpert (MTB/RIF) and 5 days for LPA [13].

Figure 1. Sample processing flow chart.



TP: True positive; LPA: Line probe Assay; MTB: Mycobacterium tuberculosis; LJ: Löwenstein-Jensen; RIF: Rifampicin; DST: drug susceptibility test.

Xpert (MTB/RIF) does not need technical expertise to run or interpret results. Furthermore, Xpert (MTB/RIF) is easily placed in remote peripheral health settings to diagnose TB and drug resistance [14]. However, LPA has logistic challenges, and the need for expert personnel may limit its use in resource-limited countries.

The precision and performance of Xpert (MTB/RIF) and LPA as compared to DST (gold standard) for drug resistance needs to be assessed, especially in resource-limited health settings. Therefore, this comparative analysis was done to evaluate the diagnostic performance characteristics of Xpert (MTB/RIF) and LPA to detect drug resistance in pulmonary tuberculosis (PTB) and extrapulmonary tuberculosis (EPTB) samples. The outcomes of this study will be important for general practitioners working in primary care, as they are the primary care providers for TB. Therefore, rapid diagnosis of drug-resistant tuberculosis and early referral for treatment are critically needed to halt the spread of MDR-TB within the population.

Methodology

Study design and laboratory setting

This cross-sectional study was conducted at the Institute of Microbiology and Molecular Genetics (MMG), University of Punjab Lahore, in collaboration with Citi Laboratories and Research Center, Lahore and Jinnah Hospital, Lahore, Pakistan. A total of 389 different clinical samples of PTB (n = 279) and EPTB (n = 110) from suspected TB cases were collected from January 2020 to December 2021. This study was approved by the Ethical Review Board of the University of Punjab, Lahore (Ethical approval number: 9043 (dated 14-11-2019). In accordance with the Declaration of Helsinki, informed consent was obtained from the recruited study participants prior to data collection.

Sample collection criteria

Early sputum samples were collected in sterile containers from all patients suspected of having PTB with their consent. In the case of EPTB, patients visited the Outpatient Door (OPD) department of Jinnah Hospital, Lahore, with clinical symptoms were referred to the histopathology department; thus, under the supervision of a qualified histopathologist, EPTB samples were collected and submitted for laboratory investigations. Every specimen was divided into three aliquots, one each for GeneXpert (MTB/RIF), LJ culture and LPA (Figure 1).

LJ (Löwenstein–Jensen medium) culture

All clinical specimens were subjected to LJ culture according to Petroff's method. In this method, samples were decontaminated, and aliquots were incubated on LJ medium at 37°C for up to 8 weeks [15]. The cultures were checked weekly for growth of MTB, and strains grown on LJ were tested for susceptibility to first-line drugs, i.e. isoniazid (INH) and rifampicin (RIF), and second-line drugs [16,17].

GeneXpert (MTB-RIF)

For GeneXpert (MTB/RIF) analysis, samples were mixed and decontaminated with the buffer provided in the kit in a 2:1 ratio (Cepheid, Sunnyvale, CA, USA). This prepared mix was manually agitated, incubated for 15 minutes at room temperature, and transferred to the Xpert (MTB/RIF) cartridge, which was inserted into the Xpert (MTB/RIF) instrument; results were automatically read after 90 minutes [17].

Drug-susceptibility test (DST)

Drug resistance in *MTB*-complex was detected by the proportional DST method on LJ [18]. This method determined the number of *MTB* colonies with defined inoculum on a controlled culture-free medium versus growth on culture media with specific critical concentration or TB drugs [18]. The first reading was done at 4 weeks. In the case of resistance, no growth was observed and re-incubation was continued for up to 42 days [19].

Line probe assay (LPA)

LPA, a strip-based molecular technology, was used to detect *MTB* DNA along with RIF and INH resistance due to *rpoB*, *inhA* and *katG* mutations. GenoType MTB-DR plus VER 2.0 kit was used to perform the LPA assay (Hain Life Sciences, Nehren, Germany) according to the manufacturer's protocol [20], LPA was conducted in three steps: DNA extraction, amplification, and hybridization [21]. Interpretation was accomplished according to the manufacturer's instructions. For PCR reaction (total volume: 50 µL), 35 µL of a primer-nucleotide master mixture (included with the kit), 10 µL of buffer containing Taq DNA polymerase and $MgCl_2$, and 5 µL of extracted DNA were used. This step was followed by 15-minute denaturation at 95°C, and two-step amplification: first step: 30 seconds at 95°C and 120 seconds at 65°C for 10 cycles; second step: followed by 25 seconds at 95°C, 40 seconds at 50°C, and 40 seconds at 70°C for 30 cycles. The final extension was for 8 minutes at 70°C. TwinCubator (Hain Life Sciences) was used for hybridisation and detection [11].

Data analysis

All categorical variables were recorded as percentages (number or frequency). Moreover, quantitative variables were represented as mean and standard deviation (SD). The diagnostic accuracy of Xpert (MTB/RIF) and LPA were analysed by calculating sensitivity, specificity, and negative and positive predictive value (NPV and PPV) by comparing it with the gold-standard test (LJ) (95% confidence interval, CI). The concordance and discordance rates of results were calculated by making 2 × 2 tables using the standard formula for all results [21]. The agreement between different tools was calculated by applying Cohen's kappa test. A test having a p value ≤ 0.01 was considered to be significant [11].

Results

Demographic profile of study participants

A total of 389 clinical samples (pulmonary and extrapulmonary) from patients with a strong suspicion of having TB were selected and processed for LJ culture, Xpert (MTB/RIF) and LPA by the proportional sampling method. Of these processed 389 samples, 59 were excluded for the following reasons: 22 were contaminated on culture; 12 had growth other than *MTB*; 10 were invalid; 10 showed error; and 5 were declared indeterminate on Xpert (MTB/RIF) (Figure 1). Out of total (n=389) samples comprised of pulmonary (n = 279) and extrapulmonary (n = 110) samples, only 320 culture positive samples were selected for final analysis. Of 320 samples, 180 (56.2%) and 140 (43.7%) were positive and negative on LJ culture, respectively. Only those samples that were also positive on LJ culture were considered "true positive" (TP). Therefore, only 180 culture positive (TP) samples were obtained for DST to evaluate drug resistance in *MTB*. Of these 180 culture-positive samples, 120 (66%) were from males and 60 (33.3%) were from females. The mean age among study participants was 35.9 years (SD = 15 years). Of these 180 culture-positive samples, 100 (55.5%) were pulmonary, and 80 (44.4%) were EPTB samples, respectively (Table 1).

Diagnostic efficacy of GeneXpert (MTB/RIF) and LPA for detection of TB

Out of a total of 320 non-duplicated clinical isolates subjected to different diagnostic techniques, 180 (55.5%) yielded growth of *MTB* on LJ culture, 165 (51.5%) detected *MTB* on Xpert (MTB/RIF) and 174

Table 1. Demographic (age, gender, sample type) distribution of study participants.

	Pulmonary (n = 100) (55.5%)	Extrapulmonary (n = 80) (44.4%)	Total (n = 180)
Age group (years)			
< 20	15	7	22 (12.2%)
21-40	56	48	104 (57.7%)
41-60	24	20	44 (24.4%)
> 60	5	5	10 (5.5%)
Gender			
Female	30	30	60 (33.3%)
Male	70	50	120 (66.6%)

Table 2. Diagnostic performance of GeneXpert and LPA for detection of MTB.

	LJ culture		
	MTB detected (n=180)	MTB non detected (n=140)	Total (n=320)
Xpert (MTB/RIF)			
MTB detected	160 (TP)	5 (FP)	165 (51.1%)
MTB not detected	20 (FN)	135 (TN)	155 (48.3%)
LPA			
MTB detected	172 (TP)	2 (FP)	174 (54.3%)
MTB not detected	8 (FN)	138 (TN)	146 (45.3%)
For LPA	Sensitivity 94.4%	Specificity 98.6%	

TP: True positive; TN: True Negative; FP: False positive; FN: False Negative; MTB: *Mycobacterium tuberculosis*; LPA: Line probe Assay.

Table 3. Overall diagnostic performance of GeneXpert (MTB/RIF) and LPA against for detection of MTB.

	Sensitivity (%)	Specificity (%)	Predictive values (%)		Kappa
			Positive	Negative	
GeneXpert	88.8	96.6	96.6	87.9	0.55 ($p < 0.01$)
LPA	94.4	98.4	98.6	94.5	0.61 ($p < 0.01$)

TP: True positive; TN: True Negative; FP: False positive; FN: False Negative; MTB: *Mycobacterium tuberculosis*; LPA: Line probe Assay; DST: Drug susceptibility testing; RIF: Rifampicin.

Table 4. Detection of RIF-mono - Resistance by Xpert (MTB/RIF) and LPA against compared to DST.

	DST-RIF		
	Resistance (n =52)	Susceptibility (n = 128)	Total (n = 180)
Xpert (MTB/RIF)			
Resistance	38 (TP)	3 (FP)	41
Susceptibility	14 (FN)	125 (TN)	139
For Xpert (MTB/RIF)	Sensitivity 73.5%	Specificity 97.0%	
LPA			
Resistance	44 (TP)	2 (FP)	46
Susceptibility	8 (FN)	126 (TN)	136
For LPA	Sensitivity 84.6%	Specificity 98.4%	

TP: True positive; TN: true negative; FP: false positive; FN: false negative; TP: True positive; TN:True Negative; FP: False positive; FN: False Negative; MTB: *Mycobacterium tuberculosis*; LPA: Line probe Assay; DST: Drug susceptibility testing; RIF: Rifampicin.

Table 5. Overall diagnostic performance of Xpert (MTB/RIF) and LPA against for detection of RIF mono resistance.

	Sensitivity (%)	Specificity (%)	Predictive value (%)		Kappa
			Positive	Negative	
Xpert (MTB/RIF)	73.3	97.6	92.6	89.9	0.65 (p <0.01)
LPA	84.6	98.4	95.6	94.5	0.80 (p <0.01)

(54.3%) were diagnosed with TB by LPA (Figure 1, Table 2). To calculate the diagnostic efficacy of Xpert (MTB/RIF) and LPA against TB, 180 culture positives were selected. Out of 180 culture-positive (TP) cases, 160 were correctly detected by Xpert (MTB/RIF), and 172 were reported positive by LPA for TB (Table 2). The calculated sensitivity and specificity for Xpert (MTB/RIF) assays were 88.8% and 96.4%, respectively. However, for TB diagnosis, the LPA showed sensitivity and specificity of 94.4% and 98.6%, respectively. The overall diagnostic performance of Xpert (MTB/RIF) and LPA for MTB detection was calculated by kappa coefficient. The calculated kappa values for Xpert (MTB/RIF) and LPA were 0.55 ($p < 0.01$) and 0.61 ($p < 0.01$), respectively (Table 3).

Performance of GeneXpert (MTB/RIF) and LPA for drug resistance

Of 320 samples tested, only 180 culture-positive isolates were subjected to DST against the first-line drugs rifampicin (RIF), ethambutol (EMB), isoniazid (INH) and pyrazinamide (PZA) and the second-line drugs (ofloxacin and kanamycin). On DST, 47 (26.5%), 52 (28.0%), 60 (33.3%), and 25 (13.5%) were resistant to PZA, RIF, INH, and ofloxacin, respectively. All strains showed 100% sensitivity to kanamycin and ethambutol. No strains were found resistant to all drugs. Not detected unpaired cases on all three parameters were excluded to determine the diagnostic accuracy of Xpert (MTB/RIF) and LPA for RIF resistance. With Xpert (MTB/RIF), 41/180 positive cases were resistant and 139/180 were sensitive to RIF, and with LPA, 46/180 strains were resistant and 134/180 were sensitive to RIF (Table 4).

DST testing was used as the gold standard for determining RIF resistance. LPA and Xpert (MTB/RIF) had sensitivity values of 97.3 % and 98.4%, respectively. Statistical analysis of study data showed moderate agreement (kappa 0.65, $p < 0.01$) (sensitivity = 73.4%, specificity = 97.3%) between Xpert (MTB/RIF) and DST for detection of RIF-mono resistance. Moreover, a perfect agreement (kappa 0.80, $p < 0.01$) was observed between LPA and DST (sensitivity = 84.4% and specificity = 98.4%) (Table 5).

Discussion

MDR-TB diagnosis has become more challenging due to its resurgence and rapid transmission. Therefore, laboratory scientists should be focused on developing an efficient and prompt diagnostic tool that helps initiate correct therapy to halt the spread of the disease [22]. The initiation of anti-TB therapy, without knowing the type of mutation in the TB strain, would result in the development of drug-resistant TB [23]. Delays in the diagnosis of MDR-TB exacerbates both disease transmission and drug resistance, resulting in an increase mortality rate [24]. Therefore, it is crucial to develop rapid and precise diagnostic tools to interrupt MDR-TB transmission and support better management strategies to avoid treatment with ineffective drugs [25]. It also prevents the unnecessary cost of administration and the existence of life-threatening side effects of second-line anti-TB drugs where the sensitive *MTB* strain is incorrectly diagnosed as being resistant [26].

This study aimed to evaluate the performance and correlation between LPA and Xpert (MTB/RIF) in detecting drug-resistant TB in Pakistan, in terms of speed of detection and drug-resistance status. Implementing these prompt diagnostic approaches will help practitioners to manage and monitor MDR-TB status by starting effective anti-TB therapy promptly.

Using LJ culture as the gold standard for TB diagnosis, LPA had a better detection threshold than Xpert (MTB/RIF) for detecting MTB in pulmonary and non-pulmonary samples. LPA had an overall diagnostic accuracy of 95.6% (kappa 0.61, $p < 0.01$), sensitivity of 94.4%, specificity of 98.4%, PPV of 98.6% and NPV of 94.5%. Meanwhile, Xpert (MTB/RIF) had a diagnostic detection rate of 83.9% (kappa 0.55, $p < 0.01$), sensitivity of 88.8%, specificity of 96.7%, positive predictive values of 96.6% and negative predictive values of 87.9% (Table 4). Comparable sensitivity and specificity of 98.4% and 66.0% by LPA and sensitivity and specificity of 78.5% and 64.3% by Xpert (MTB/RIF) were previously reported in a 2019 African study [11]. The outcomes of previously published similar studies, where the sensitivity and specificity of LPA and Xpert (MTB/RIF) were calculated for the diagnosis of TB, showed agreement with our findings [27,28].

The study was designed to evaluate the correlation between Xpert (MTB/RIF) and LPA for detecting RIF-mono-resistant strains of *MTB*. RIF, which is one of the first lines of drugs used for the treatment of TB, was discovered in 1965 [29]. It's sterilizing activity and ability to shorten treatment duration make it one of the most important drugs used for TB treatment [29]. However, the emerging trend of *MTB* developing resistance against RIF makes therapy challenging. Culture-based conventional microbiology techniques (DST) are time- consuming and complex and therefore not ideal in resource-limited countries. WHO has recommended nucleic-acid-based LPA and GeneXpert (MTB/RIF) tests for rapid drug resistance [24].

In our study, we compared LPA and Xpert (MTB/RIF) with DST for detecting RIF-mono resistance. The diagnostic agreement between LPA, Xpert (MTB/RIF) and DST was determined by calculating the kappa coefficient. We observed that GeneXpert attained reasonable agreement (kappa 0.65, $p < 0.01$), while LPA achieved high agreement (kappa 0.81, $p < 0.01$) for the detection of RIF resistance. LPA showed a diagnostic sensitivity of 84.3% compared to 73.6% with Xpert (MTB/RIF) (Table 4). In Kenya, a similar comparative analysis between LPA and Xpert (MTB/RIF) was done to evaluate the diagnostic accuracy of these methods for the detection of MDR-TB [11]. The outcome of their analysis endorsed our finding where they reported a moderated agreement (kappa 0.59, $p < 0.01$) by Xpert (MTB/RIF); however, LPA had a high agreement (kappa 0.89, $p < 0.01$) for the detection of RIF-mono resistance [11]. The superiority of LPA, with a sensitivity of 95.2% (kappa 0.853, $p < 0.001$), over Xpert (MTB/RIF), with the sensitivity of 84.9% (kappa 0.621, $p < 0.001$), for detection of RIF-mono resistance was also reported in TB-endemic areas of Africa [12]. A report from Taiwan described the early detection of MDR-TB cases among high-risk populations by using LPA – a sensitivity of 70.7% and a specificity of 65.7% was reported [25]. Moreover, the diagnostic accuracy for RIF-mono-resistance was 96.5% [26]. The results of the published Indian study are also in agreement with our results, where they showed 100% agreement for LPA, but for Xpert (MTB/RIF), they showed only 64.4% agreement [30]. The other published data also showed a similar trend between LPA and Xpert (MTB/RIF) for the detection of RIF-mono resistance [27,31,32]. The results of the present study indicated that molecular-based diagnostic approaches were comparable and consistent with the conventional gold-standard DST method for the detection of MDR-TB. A possible reason for the low agreement between GeneXpert (MTB/RIF) and DST for detection of RIF-mono resistance may be the discrepancy in volume of sample discharged into Xpert (MTB/RIF) cartridge [21, 33].

Hence, based on our study outcomes we recommend using either GeneXpert or LPA for detecting RIF mono-resistant or MDR-TB. However, LPA showed superiority over Xpert (MTB/RIF) in terms of its diagnostic accuracy for the detection of TB and MDR-TB. From an operational perspective and in terms of detection time, Xpert (MTB/RIF) has significant advantages over LPA. Xpert (MTB/RIF) is user-friendly, with a short turnaround time (TAT) of 2–3 hours compared to LPA, which took 2–3 days. Moreover, GeneXpert (MTB/RIF) does not require skilled lab personnel to perform as is the case for LPA. Therefore, the Xpert (MTB/RIF) assay worked well in low-resource health settings. Thus, due to LPA's operational issues, its implementation as a diagnostic tool for detecting RIF mono resistance, especially in burden countries with low resources, is still under debate [34]. Furthermore, geographical variations, the selected population's TB incident rate, and the diverse nature of *rpoB* gene mutation could be attributed to discrepancies in the diagnostic performance of LPA and Xpert (MTB/RIF) [35]. Moreover, due to their high sensitivity, specificity, and short turnaround time, both molecular tools may be preferred over conventional methods, but have their own drawbacks. Xpert (MTB/RIF) only picks up RIF resistance, which, if used as a proxy for MDR-TB and not INH resistance is not determined by Xpert (MTB/RIF), can cause MDR-TB to be overestimated [36]. Such situations might not require complete MDR-TB therapy. LPA has complex laboratory infrastructure drawbacks that prevent its use in underdeveloped countries.

Study limitations

There are some limitations. First, the sample size was small. Secondly, samples from control patients (healthy population) were not available to serve as negative control. Third, this study included only those patients who visited Jinnah Hospital, Lahore, for treatment. Therefore, the data may not truly represent Pakistan's general population. Thus, the outcomes of this study should be interpreted with these limitations.

Conclusions

Using LPA and Xpert (MTB/RIF) alone or together would have a significant impact on the rapid detection of drug-resistant TB in Pakistan due to their comparable sensitivity and specificity with DST. Implementing both methods could help TB clinicians by detecting different mutations associated with drug resistance quickly. It is never feasible to set up conventional DST laboratories for the detection of drug-resistant TB in resource-limited settings. Therefore, Xpert (MTB/RIF) and LPA have provided diagnostic relief to TB clinicians by providing a rapid clear status of RIF-mono resistant in TB strains, which can ultimately lead to better patient outcomes and help control the spread of drug-resistant TB in the country.

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