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Redefining MTBDR_{plus} test results: What do indeterminate results actually mean?

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Abstract

Early diagnosis of drug-resistant tuberculosis is critical in order to establish appropriate drug treatment regimens and to prevent the further transmission of resistant strains. Line probe assays (LPAs) are promising rapid diagnostics for use in low income settings, and can be used to screen smear-positive sputum samples for resistance to rifampicin (RIF) and isoniazid (INH) in as little as 1–2 days. Though the diagnostic performance of these assays has been well evaluated, little research has elucidated the true nature of indeterminate LPA results, or assessed the ability of these assays to perform on a wide range of clinical samples

We evaluated the performance of the commercially available Genotype MTBDR_{plus} LPA against conventional MGIT 960 culture and drug susceptibility testing (DST) results for 308 pulmonary (PTB) and 32 extrapulmonary (EPTB) tuberculosis samples. Invalid LPA results (defined as missing the MTB identification band) were obtained for 18 PTB samples, which were excluded from further analysis. The sensitivity and specificity of the Genotype MTBDR_{plus} assay for multidrug-resistant tuberculosis (MDR-TB), based upon the results obtained for the remaining 322 samples, was 95.2% and 95.1%, respectively. 13.7% (40/290) of the PTB samples were not interpretable, or indeterminate, by LPA (defined as having the absence of both wild type and corresponding mutation bands) for INH and/or RIF and were further evaluated by pyrosequencing (PSQ). Contrary to standard LPA interpretation, INH and RIF susceptibility were confirmed by both DST and PSQ in 7.5% (3/40) and 27.5% (11/40) of indeterminate samples, respectively.

When LPA test results were not interpretable in this study, PSQ was found to be a valuable and rapid technique to resolve discrepancies.

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Keywords

Tuberculosis; Drug Resistance; Pyrosequencing; Heteroresistance; Indeterminate

Introduction

Tuberculosis (TB) remains one of the world's deadliest communicable diseases. The recent increase of drug-resistant strains, as a result of the ineffective treatment and increased direct transmission of resistant infections, presents an important dilemma for global TB control efforts [1]. The detection of resistance is often delayed when utilizing conventional phenotypic drug susceptibility testing (DST) methods due to the slow growth of *Mycobacterium tuberculosis* (MTB) in liquid culture. These conventional growth-based diagnostic methods require approximately four weeks to generate results [3]. During this time, patients may be taking medications that are completely ineffective, and risk directly transmitting resistant infections unto others. As such, there is an urgent need for rapid diagnostic tests for drug-resistant TB strains.

MTB acquires drug resistance through the accumulation of resistance-associated mutations on specific genes [2, 3]. The World Health Organization (WHO) has recently approved a commercially available line probe assay (LPA), the Genotype MTBDR*plus* assay [Hain Lifescience], which detects drug-resistance based upon the presence or absence of these specific resistance-associated mutations [4]. The diagnostic performance of the Genotype MTBDR*plus* assay is based upon the amplification and genetic analysis of those gene regions known to harbor resistance-associated mutations, as determined by reverse hybridization to wild type and mutated sequences. The assay detects the presence of MTB as well as the most common genetic mutations conferring resistance to RIF (mutations within the *rpoB* gene) and INH (mutations within the *katG* gene and *inhA* promoter) [5, 6], and its diagnostic performance has been well evaluated in pulmonary samples [40]. In this study, the Genotype MTBDR*plus* diagnostic LPA was thoroughly evaluated for its ability to detect multidrug-resistant TB (MDR-TB) in pulmonary (PTB) and extrapulmonary (EPTB) samples as compared to standard DST methods. Any discrepancies were further evaluated at the molecular level using pyrosequencing (PSQ), a real time sequencing method, in order to comment upon the true nature of indeterminate and discrepant test results.

Materials and Methods

Setting

The study was performed at P. D. Hinduja National Hospital and Medical Research Center, Mumbai, from August 2011– May 2013. The Mycobacteriology laboratory at Hinduja Hospital is accredited through the College of American Pathologists (CAP), the National Accreditation Board for Testing and Calibration Laboratories (NABL), and the Central TB Division, Government of India (CTD, GOI), for performance of liquid culture and DST.

Study Design and Ethics Statement

This single site, cross-sectional study was conducted in order to determine the diagnostic performance of the Genotype MTBDR*plus* assay for PTB and EPTB samples, in comparison to conventional methodologies. Indeterminate results were further evaluated by pyrosequencing.

The study was approved by the Institutional Review Board (IRB) of P. D. Hinduja National Hospital and Medical Research Center (National Health and Education Society), Mumbai, India. Consent was waived for this study, as the study was to be performed on existing sediments identified only by laboratory-generated numbers and with no traceability back to the patients. All patient details remained confidential.

Sample Collection

A total of 340 consecutive samples were previously collected. Both phenotypic and genotypic testing was conducted on 308 PTB samples (sputum) and 32 EPTB samples (pus, body fluid, biopsy, tissue etc.).

Ziehl-Neelsen Staining

All collected samples were graded as per WHO recommended criteria [7]. EPTB samples selected for the present study were all smear positive, as recommended for MTBDR*plus* assay.

Sample Processing

All samples were processed by the NALC-NaOH method, as described previously [8]. Final sediments were resuspended in 2ml of PBS (pH= 7.4). Of this suspension, 500 µl was utilized to inoculate a 7ml MGIT tube and 500 µl was used to inoculate solid media (LJ). The remaining sediment was transferred to 1.5ml screw cap tubes for further genotypic analysis. Sediments were stored at –80°C when not in use.

Drug Susceptibility Testing (DST)

Positive liquid MGIT 960 cultures were subjected to further phenotypic DST testing. The drug concentrations used to determine RIF and INH susceptibility were 1µg/mL and 0.1µg/ml, respectively. [28]

Genotypic MTBDR*plus* Assay

DNA was extracted from 500µl of each decontaminated patient sediment sample. All DNA samples were stored at –80°C when not in use. The Genotype MTBDR*plus* assay was performed for each sample as per manufacturer's instructions [9]. The assay contains six control probes: a conjugate control, an amplification control, an MTB complex control, and *rpoB*, *katG* and *inhA* amplification controls. For the detection of INH and RIF resistance, the assay strip contains 21 mutation and wild type probes: eight *rpoB* wild-type probes (covering codons 505 to 533); the four *rpoB* mutant probes D516V, D526Y, H526D, and S531L; a *katG* codon 315 wild-type probe; two *katG* codon 315 mutant probes with AGC-ACC and AGC-ACA mutations; two *inhA* wild-type probes covering positions –15 and –16

of the gene regulatory region; and four *inhA* mutant probes with mutations C→T at position –15, A→G at position –16, T→C at position –8, and T→A at position –8. A detailed test flow chart for this study is shown in Figure 1. Assay results were considered invalid if the control probes were absent, and not interpretable (NI)/indeterminate if the gene loci probe for both the wildtype and mutation probes being assessed was absent. The MTBDR*plus* assay was not repeated if results were NI/indeterminate.

Pyrosequencing (PSQ)

PSQ was performed on all samples found to be NI by MTBDR*plus*. The same extracted DNA sample, as used for the line probe assay, was used for PSQ. The PSQ assay utilized in this study involved three essential parts: amplification of gene fragments by PCR, capture of biotinylated single-stranded DNA on streptavidin sepharose beads, and sequencing by a commercially available, modified PSQ platform [10]. Our PSQ assay included eight sub-assays: one for identification of MTB and seven for detection of drug resistance mutations. For determination of INH resistance, the assay sequences three loci: *katG* codons 312–316, the *inhA* promoter from –4 to –20, and the *ahpC* promoter from –4 to –23. Mutations in the *ahpC* promoter are believed to be compensatory to mutations in *katG*, which cause deficient catalase activities, and are indirectly associated with INH resistance [24, 25]. The molecular target included in the PSQ assay for determination of RIF resistance is the RIF-resistance determining region of the *rpoB* gene (codons 507–533).

Statistical Analysis

Diagnostic tests were evaluated as per STARD recommendations [11]. Sensitivity and specificity were calculated by comparing phenotypic and molecular test results using Meta Disc software.

Results

Concordance between LPA and DST Results

A total of 322 samples were analyzed by Genotype MTBDR*plus* assay and phenotypic DST, after excluding 18 samples wherein the MTB band was not detected by the molecular test. The average turnaround time for LPA within the context of our study was 2–3 days (data not shown). Among the 322 samples available for analysis, 290 were PTB and 32 were EPTB. According to phenotypic DST, 65% (211/322) of these were MDR and 22.3% were sensitive to both RIF and INH. Phenotypic monoresistance to RIF and monoresistance to INH was seen for 2.6% (8/322) and 10% (32/322) of samples, respectively.

After excluding three samples failing to detect the MTB band, as above, the genotypic LPA assay found 40 samples to be NI, showing the absence of both wild type and mutation probes for INH and/or RIF resistance detection (Table 1). As such, LPA data was available for both RIF and INH in 282 samples, as shown in Table 2. Out of the total 322 samples evaluated by LPA, 59.2% (180/282) were confirmed to be MDR and 27.6% (78/282) were found to be sensitive to both RIF and INH (Figures 2 and 3). Monoresistance to RIF or INH was found in 1.7% (5/282) and 6.7% (19/282) of samples, respectively, for samples with interpretable genetic data for both drug compounds.

Not Interpretable (NI)/Indeterminate Genotypic Test Results

NI/indeterminate results occurred concurrently for RIF and INH in 2.4% (8/322) of tests. RIF^{NI} occurred in 9.6% (29/322) of tests and INH^{NI} occurred in 4.3% (14/322) of tests. Follow-up PSQ analysis showed that, among the eight concurrent RIF^{NI} and INH^{NI} results, relevant resistance-associated mutations were indeed present in 7/8 samples. The remaining sample, though phenotypically resistant for INH, was found to be wild type for relevant gene regions by PSQ. Among the 14 solely INH^{NI} results encountered, PSQ detected mutations in 12/13 samples that were phenotypically resistant to INH, and 1 DST INH-resistant sample was found to be wild type. The one DST INH-susceptible sample was found to be wild type by PSQ. Of the 29 RIF^{NI} encountered, 20 samples were phenotypically resistant to RIF and 9 samples were phenotypically susceptible. PSQ confirmed mutations in all 20 RIF-resistant samples while the 9 RIF-susceptible samples were all found to have wild type *rpoB* sequences. Compared to phenotypic DST, genotypically discordant results occurred a total of 46 times [INH (27) and RIF (19)], as examined in Table 2.

Detection of Mutations associated with Drug Resistance

The *rpoB* Mutant 3 (S531L) was the predominant mutation found in PTB (132) and EPTB (18) among the 172 RIF resistant samples evaluated by LPA, as shown in Table 3. For the 200 INH resistant samples evaluated, the *katG* Mutant 1(315 AGC-ACC) was the predominant mutation identified amongst both PTB (153) and EPTB [18] samples, as shown in Table 4.

Discussion

This study investigated the full performance of the Genotype MTBDR^{plus} assay, assessing both interpretable and non-interpretable results for a range of PTB and EPTB samples. The sensitivity of the assay was found to be suboptimal in the smear negative, culture-positive sputum samples and smear positive EPTB samples evaluated. The sensitivity of the LPA for MDR-TB was 95.29%; with a and specificity was 95.16%. The sensitivity for the detection of INH resistance was 89.29%, with a specificity of 95.95%. However, the Genotype MTBDR^{plus} assay failed to detect 8.05% of INH resistant strains. The sensitivity for detection of RIF resistance in our study was 91.98%, with a specificity of 95.79%, but the assay failed to detect 5.31% of RIF resistant strains. The S531L mutation in the *rpoB* gene was the most frequently occurring mutation among RIF resistant strains (n=162) [37, 38, 39] as observed in previous studies. For determination of INH resistance, the S315T mutations in the *katG* gene were by far the most common found in our study (n=196).

Recent studies have found the MTBDR^{plus} assay to be an effective and feasible diagnostic tool for MDR-TB screening in endemic regions [12, 13, 14, 24, 42]. While the sensitivity of the molecular diagnostic test for detection of INH resistance in our study was slightly lower than that for RIF and MDR detection (compared to conventional methods), it was analogous to that of previously reported studies [25, 31, 32]. The slightly lower sensitivity of the MTBDR^{plus} test for INH, as compared to conventional methods, is likely due to the presence of genetic mutations conferring INH resistance that are located outside the *katG* and *inhA* promoter gene regions included in the LPA [26, 27, 33, 35]. In the present study,

we did not identify any *ahpC* promoter mutations via PSQ, but other gene regions have been implicated in conferring resistance to INH that were not included in the assay, such as *kasA* [41]. An alternative explanation for the persistence of discordant results may be the presence of mixed populations of bacteria, consisting of both susceptible and resistant strains, and hetero-resistance [29, 30, 34]. In the present study, 6.2% (18/290) of samples were considered hetero-resistant by LPA, and were phenotypically resistant by MGIT DST. Pyrosequencing confirmed these samples to be genotypically resistant, indicating that observed discrepancies were likely due to signal overdevelopment during LPA, rather than mixed infection. As the MTBDRplus assay is currently only recommended for smear positive samples, having a poor limit of detection for MTB, the detection of heteroresistance is unlikely.

LPA indeterminate have been previously reported to be related to smear- and culture-status of the evaluated samples, and generally occur in 1.4–19.2% of samples, depending on sample type (EPTB v. PTB), [40] with un-interpretable reads only occurring in 6% of smear positive sputum samples upon test repeat [42]. Our study found 12.4% of results to be NI without test repeat. According to the manufacturer's instructions for reporting of LPA results, samples demonstrating the absence of wild type and corresponding mutation bands (NI/Indeterminate results) are to be reported as resistant. Although this interpretation proved accurate for the majority of INH^{NI} and RIF^{NI} results in our study, other LPA indeterminate results from this study were not as easily resolved, highlighting an important limitation for MTBDRplus performance. In the present study, NI results were proven susceptible by DST and PSQ in a notable 7.5% (3/40) and 27.5% (11/40) of INH^{NI} and RIF^{NI} samples, respectively. Although the molecular basis of these discordant LPA results has not been fully elucidated, we can speculate as to the mechanisms behind test discordance. The occurrence of NI results where the wild type probes failed to hybridize to their corresponding sequences could result from the inaccessibility of the genomic sequence in a given sample, such as when secondary DNA structures are present. This problem has been noted in rpoB hybridization, in particular, and might play a role in the observed high occurrence (27.5%) of NI results proven RIF-susceptible in this study [36].

Conclusions

Although the Genotype MTBDRplus test has proven to be a reliable, rapid and easy diagnostic for the simultaneous detection of RIF and INH resistance in *M. tuberculosis*, the nuances of LPA performance in regards to the occurrence and interpretation of indeterminate results are still being evaluated. This study has found the conventional interpretation of LPA indeterminate results as resistant to be incorrect 7.5% of the time when assessing INH resistance and 27.5% of the time when assessing RIF resistance. In case of discrepant results, we find that PSQ can be a valuable tool to rapidly evaluate LPA indeterminate results, in lieu of repeating phenotypic DST, as the technology appears to resolve discrepancies in line with phenotypic DST results.

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What is the key question?

Does the absence of wild type and corresponding mutation probes on an LPA strip actually indicate resistant strains, as per manufacturer's instructions?

What is the bottom line?

Clinicians should correlate discrepant LPA results.

Why read on?

To decipher indeterminate LPA results.

Figure 1.

Flow Chart of Sample Testing Process and Phenotypic and Genotypic Testing Results.

MTB= *Mycobacterium tuberculosis*, DST= drug susceptibility testing, PTB= pulmonary tuberculosis, EPTB= extrapulmonary tuberculosis, R= Rifampicin, H= Isoniazid, ^R = Resistant, ^S = Susceptible.

Figure 2.

Specificity Forest Plot.

DST= Drug susceptibility testing, LPA= Line Probe Assay, P= pulmonary tuberculosis, EP= extrapulmonary tuberculosis, R= Rifampicin, H= Isoniazid

Figure 3.

Sensitivity Forest Plot.

DST= Drug susceptibility testing, LPA= Line Probe Assay, P= pulmonary tuberculosis, EP= extrapulmonary tuberculosis, R= Rifampicin, H= Isoniazid

Table 1

Comparison of Phenotypic DST and PSQ Results for Samples Found to be Not Interpretable (NI)/Indeterminate by Genotype MTBDR*plus* Assay.

		INH ^{NI} (n= 14)		RIF ^{NI} (n= 29)	
		PSQ		PSQ	
		R	S	R	S
DST	R	12	1	20	0
	S	0	1	0	9

R= Resistant

S= Susceptible

NI= Not Interpretable

INH= Isoniazid

RIF= Rifampicin

PSQ= Pyrosequencing

DST= Drug Susceptibility Testing

Table 2

Comparison of Line Probe Assay (LPA) and Phenotypic DST Results for RIF and INH, excluding Not Interpretable (NI) LPA Results.

		INH (n=298)		RIF (n=282)	
		DST		DST	
		R	S	R	S
LPA	R	200	3	172	4
	S	24	71	15	91

R= Resistant

S= Susceptible

NI= Not Interpretable

INH= Isoniazid

RIF= Rifampicin

LPA= Line Probe Assay

DST= Drug Susceptibility Testing

Table 3

LPA Mutation Findings and Distribution for Molecular Evaluation of RIF Susceptibility.

LPA Result	Mutation	PTB No. of Mutants	EPTB No. of Mutants
Mutant 1	D516V	5	0
Mutant 2A	H526Y	2	0
Mutant 2B	H526D	3	0
Mutant 3	S531L	144	18

LPA= Line Probe Assay

PTB= Pulmonary Tuberculosis

EPTB= Extrapulmonary Tuberculosis

Table 4

LPA Mutation Findings and Distribution for Molecular Evaluation of INH Susceptibility.

LPA Result	Mutation	PTB No. of Mutants	EPTB No. of Mutants
<i>katG</i> Mutant 1	AGC-315-ACC	153	18
<i>katG</i> Mutant 2	AGC-315-ACA	4	0
<i>katG</i> Mutant 1 & <i>inhA</i> Mutant 1	AGC-315-ACC & -15C/T	16	5
<i>inhA</i> Mutant 1	-15C/T	0	0
<i>inhA</i> Mutant 2	-16A/G	0	1
<i>inhA</i> Mutant 3A	-8T/C	0	0
<i>inhA</i> Mutant 3B	-8T/A	3	0

LPA= Line Probe Assay

PTB= Pulmonary Tuberculosis

EPTB= Extrapulmonary Tuberculosis