Redefining MTBDRplus test results: what do indeterminate results actually mean?

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SUMMARY

BACKGROUND: Although line-probe assays (LPAs) are promising, little research has been conducted to elucidate the true nature of indeterminate LPA results or assess the ability of these assays to perform on a wide range of clinical samples.

OBJECTIVE: To evaluate the performance of the commercially available GenoType® MTBDRplus LPA against conventional BACTEC™ MGIT™ 960 culture and drug susceptibility testing (DST) among 308 pulmonary tuberculosis (PTB) and 32 extra-pulmonary TB samples.

RESULTS: Invalid LPA results (defined as those with a missing Mycobacterium tuberculosis identification band) were obtained for 18 PTB samples, which were excluded from further analysis. The sensitivity and specificity of the MTBDRplus assay for multidrug-resistant TB, based upon the results obtained for the remaining 322 samples, was respectively 95.2% and 95.1%. Of 290 PTB samples, 40 (13.7%) were indeterminate on LPA (defined as the absence of both wild-type and corresponding mutation bands) for isoniazid (INH) and/or rifampicin (RMP), and were further evaluated by pyrosequencing (PSQ). Contrary to standard LPA interpretation, INH and RMP susceptibility were confirmed by both DST and PSQ in respectively 7.5% (3/40) and 27.5% (11/40) of indeterminate samples.

CONCLUSION: PSQ was found to be a valuable and rapid technique to resolve discrepancies in LPA test results that were not interpretable.

KEY WORDS: tuberculosis; drug resistance; pyrosequencing; heteroresistance; indeterminate

TUBERCULOSIS (TB) remains one of the world’s deadliest communicable diseases. The recent increase in drug-resistant strains as a result of the ineffective treatment and increased direct transmission of resistance presents an important dilemma for global TB control efforts.1 The detection of resistance is often delayed when using conventional phenotypic drug susceptibility testing (DST) methods due to the slow growth of Mycobacterium tuberculosis in liquid culture. Conventional growth-based diagnostic methods require approximately 4 weeks to generate results.2 During this time, patients may be taking medications that are completely ineffective, and risk directly transmitting resistant disease to others. There is therefore an urgent need for rapid diagnostic tests for drug-resistant TB strains.

M. tuberculosis acquires drug resistance through the accumulation of resistance-associated mutations in specific genes.2,3 The World Health Organization (WHO) has recently approved a commercially available line-probe assay (LPA), the GenoType® MTBDRplus assay (Hain Lifescience, Nehren, Germany), which detects drug resistance based on the presence or absence of these specific resistance-associated mutations. The diagnostic performance of the MTBDRplus assay is based on the amplification and genetic analysis of those gene regions known to harbor resistance-associated mutations, as determined by reverse hybridization to wild-type (wt) and mutated sequences. The assay detects the presence of M. tuberculosis as well as the most common genetic mutations conferring resistance to rifampicin (RMP) (mutations within the rpoB gene) and isoniazid (INH) (mutations within the katG gene and the inhA promoter),4,5 and its diagnostic performance has been well evaluated in pulmonary samples.4

In the present study, the MTBDRplus diagnostic LPA was thoroughly evaluated for its ability to detect multidrug-resistant TB (MDR-TB) in pulmonary (PTB) and extra-pulmonary (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, to ascertain the true nature of indeterminate and discrepant test results.

MATERIALS AND METHODS

Setting
The study was performed at the P D Hinduja National Hospital and Medical Research Centre, Mumbai, India, from August 2011 to May 2013. The Mycobacteriology Laboratory at the Hinduja Hospital is accredited by 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 the performance of liquid culture and DST.

Study design and ethics statement
This single site, cross-sectional study was conducted to determine the diagnostic performance of the MTBDRplus assay in PTB and EPTB samples in comparison with conventional methodologies. Indeterminate results were further evaluated using pyrosequencing.

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

Sample collection
A total of 340 previously collected consecutive samples were used for the study. 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 according to WHO-recommended criteria. EPTB samples selected for the study were all smear-positive, as recommended for the MTBDRplus assay.

Sample processing
All samples were processed using the N-acetyl-L-cysteine-sodium hydroxide method, as described previously. Final sediments were resuspended in 2 ml phosphate buffered saline (PBS) (pH = 7.4); 500 µl of this suspension was inoculated into a 7 ml MGIT tube and 500 µl was inoculated onto solid media (Lowenstein-Jensen). The remaining sediment was transferred to 1.5 ml screw cap tubes for further genotypic analysis. Sediments were stored at -80°C when not in use.

Drug susceptibility testing
Positive liquid MGIT 960 cultures were subjected to further phenotypic DST. The drug concentrations used to determine RMP and INH susceptibility were respectively 1 µg/ml and 0.1 µg/ml.

Genotypic MTBDRplus assay
DNA was extracted from 500 µl of each decontaminated patient sediment sample. All DNA samples were stored at -80°C when not in use. The MTBDRplus assay was performed on each sample according to the manufacturer’s instructions. The assay contains six control probes: a conjugate control, an amplification control, an M. tuberculosis complex control, and rpoB, katG and inhA amplification controls. For the detection of INH and RMP resistance, the assay strip contains 21 mutation and wt probes: eight rpoB wt probes (covering codons 505 to 533); D516V, D526Y, H526D, and S531L, the four rpoB mutant probes; a katG codon 315 wt probe; two katG codon 315 mutant probes with AGC–ACC and AGC–ACA mutations; two inhA wt 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 given in Figure 1. assay results were considered invalid if the control probes were absent, and non-interpretable (NI) or indeterminate if the gene loci probe for both the wt and mutation probes being assessed was absent. The MTBDRplus assay was not repeated if the results were NI or indeterminate.

Pyrosequencing
Pyrosequencing (PSQ) was performed on all samples found to be NI on MTBDRplus. The same extracted DNA sample as used for the LPA was used for PSQ. The PSQ assay used in this study involved three essential parts: amplification of gene fragments using polymerase chain reaction, capture of biotinylated single-stranded DNA on streptavidin sepharose beads, and sequencing by a commercially available modified PSQ platform. Our PSQ assay included eight subassays: one for the identification of M. tuberculosis and seven for the detection of drug resistance mutations. For the determination of INH resistance, the assay sequences three loci: katG codons 312-316, the inhA promoter from −4 to −20, and the abpC promoter from −4 to −23. Mutations in the abpC promoter are believed to be compensatory to mutations in katG, which cause deficient catalase activities, and are indirectly associated with INH resistance. The molecular target included in the PSQ assay for the determination of RMP resistance is the RMP resistance-determining region of the rpoB gene (codons 507–533).
Statistical analysis
Diagnostic tests were evaluated according to STARD (Standards for Reporting Studies of Diagnostic Accuracy) recommendations.13 Sensitivity and specificity were calculated by comparing phenotypic and molecular test results using Meta-DiSc software (Unit of Clinical Biostatistics, Ramón y Cajal Hospital, Madrid, Spain).

RESULTS
Concordance between LPA and DST results
A total of 322 samples were analyzed using the GenoType MTBDRplus assay and phenotypic DST, after excluding 18 samples with an undetected Mycobacterium tuberculosis band on molecular testing. The average turnaround time for LPA within the context of our study was 2–3 days (data not shown). Of the 322 samples available for analysis, 290 were PTB and 32 were EPTB. According to the phenotypic DST results, 65% (211/322) of these were MDR-TB and 22.3% were susceptible to both RMP and INH. Phenotypic monoresistance to RMP and monoresistance to INH was observed in respectively 2.6% (8/322) and 10% (32/322) of the samples.

After excluding three samples with an undetected M. tuberculosis band, as above, the genotypic LPA assay found 40 samples to be NI, showing the absence of both wt and mutation probes for INH and/or RMP resistance detection (Table 1). LPA data were thus available for both RMP and INH in 282 samples (Table 2). Of the total 322 samples evaluated using LPA, 59.2% (180/282) were confirmed to be MDR-TB and 27.6% (78/282) were found to be susceptible to both RMP and INH (Figures 2 and 3). Mono-resistance to RMP or INH was found in respectively 1.7% (5/282) and 6.7% (19/282) of samples with interpretable genetic data for both drug compounds.

<table>
<thead>
<tr>
<th>INH&lt;sub&gt;NI&lt;/sub&gt; (n = 14)</th>
<th>RMP&lt;sub&gt;NI&lt;/sub&gt; (n = 29)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSQ</td>
<td>PSQ</td>
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<tr>
<td>DST</td>
<td>R</td>
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<td>R</td>
<td>12</td>
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<tr>
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<tr>
<td></td>
<td>20</td>
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</table>

DST = drug susceptibility testing; PSQ = pyrosequencing; NI = non-interpretable; INH = isoniazid; RMP = rifampicin; R = resistant; S = susceptible.

Figure 1 Flow chart of sample testing process and phenotypic and genotypic testing results. LPA = line-probe assay; DST = drug susceptibility testing, PTB = pulmonary tuberculosis; EPTB = extra-pulmonary TB; R = rifampicin, H = isoniazid; <sup>R</sup> = resistant; <sup>S</sup> = susceptible; <sup>NI</sup> = non-interpretable.

<table>
<thead>
<tr>
<th>INH (n = 298)</th>
<th>RMP (n = 282)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DST</td>
<td>DST</td>
</tr>
<tr>
<td>LPA</td>
<td>RMP</td>
</tr>
<tr>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>200</td>
<td>71</td>
</tr>
<tr>
<td>172</td>
<td>15</td>
</tr>
</tbody>
</table>

LPA = line-probe assay; DST = drug susceptibility testing; RMP = rifampicin; INH = isoniazid; NI = non-interpretable; R = resistant; S = susceptible.
Non-interpretable or indeterminate genotypic test results

Non-interpretable or indeterminate results occurred concurrently for RMP and INH in 2.4% (8/322) of the tests. RMP\textsuperscript{NI} occurred in 9.6% (29/322) and INH\textsuperscript{NI} occurred in 4.3% (14/322) of the tests. Follow-up PSQ analysis showed that, among the eight concurrent RMP\textsuperscript{NI} and INH\textsuperscript{NI} results, relevant resistance-associated mutations were indeed present in 7/8 samples. The remaining sample, although phenotypically resistant to INH, was found to be wt for the relevant gene regions by PSQ. Among the 14 solely INH\textsuperscript{NI} results encountered, PSQ detected mutations in 12/13 samples that were phenotypically resistant to INH, and one DST INH-resistant sample was found to be wt. The one DST INH-susceptible sample was found to be wt on PSQ. Of the 29 RMP\textsuperscript{NI} results encountered, 20 samples were phenotypically resistant to RMP and 9 samples were phenotypically susceptible. Mutations were confirmed using PSQ in all 20 RMP-resistant samples, while the 9 RMP-susceptible samples were all found to have wt \(rpoB\) sequences.

Detection of mutations associated with drug resistance

\(rpoB\) Mutant 3 (S531L) was the predominant mutation found in PTB (\(n = 132\)) and EPTB (\(n = 18\)) among the 172 RMP-resistant samples evaluated using LPA (Table 3). For the 200 INH-resistant samples evaluated, \(katG\) Mutant 1 (315 AGC–ACC) was the predominant mutation identified among both PTB (\(n = 133\)) and EPTB (\(n = 18\)) samples (Table 4).

\textbf{DISCUSSION}

The present study investigated the full performance of the GenoType MTBDR\textsuperscript{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 LPA for MDR-TB was 95.29%; the specificity was 95.16%. The sensitivity for the detection of INH resistance was 89.29%, with a specificity of 95.95%. However, the MTBDR\textsuperscript{plus} assay failed to detect 8.05% of INH-resistant strains. The sensitivity for the detection of RMP resistance in our study was 91.98%, with a specificity of 95.79%; however, the assay failed to
detect 5.31% of RMP-resistant strains. The S531L mutation in the rpoB gene was the most frequently occurring mutation among RMP-resistant strains \((n = 162)\), as observed in previous studies.\(^{14–16}\) 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\textsubscript{plus} assay to be an effective and feasible diagnostic tool for MDR-TB screening in endemic regions.\(^{12,17–20}\) While the sensitivity of the molecular diagnostic test for the detection of INH resistance in our study was slightly lower than that for RMP and MDR-TB detection (compared to conventional methods), it was analogous to that of previously reported studies.\(^{12,20,21}\) The slightly lower sensitivity of the MTBDR\textsubscript{plus} test for INH than 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.\(^{4,22–24}\) In the present study, we did not identify any \(ahpC\) promoter mutations using PSQ, but other gene regions have been implicated in conferring resistance to INH that were not included in the assay, such as \(kasA\).\(^4\) 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 heteroresistance.\(^{8,25,26}\) In the present study, 6.2% \((18/290)\) of samples were considered heteroresistant on LPA and phenotypically resistant on MGIT DST. PSQ 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 MTBDR\textsubscript{plus} assay is currently only recommended for smear-positive samples because of its poor limit of detection for \(M.\) \textit{tuberculosis}, the detection of heteroresistance is unlikely.

Indeterminate LPA results have been previously reported to be related to the smear and culture status of the evaluated samples, and generally occur in 1.4–19.2% of samples, depending on sample type (EPTB vs. PTB),\(^4\) with uninterpretable readings only occurring in 6% of smear-positive sputum samples upon test repeat.\(^{20}\) Our study found 12.4% of results to be NI without repeating the test. According to the manufacturer’s instructions for reporting LPA results, samples demonstrating the absence of wt and corresponding mutation bands (NI/indeterminate results) are to be reported as resistant. Although this interpretation proved accurate for the majority of the INH\textsubscript{NI} and RMP\textsubscript{NI} results in our study, other LPA indeterminate results from this study were not as easily resolved, highlighting an important limitation of MTBDR\textsubscript{plus}.\(^{27,28}\) In the present study, NI results proved susceptible on DST and PSQ in a notable 7.5% \((3/40)\) and 27.5% \((11/40)\) of INH\textsubscript{NI} and RMP\textsubscript{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 wt 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 to be RMP-susceptible in this study.

### CONCLUSIONS

Although the MTBDR\textsubscript{plus} test has been proven to be a reliable, rapid and easy diagnostic for the simultaneous detection of RMP and INH resistance in \(M.\) \textit{tuberculosis}, the nuances of LPA performance with regard 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 RMP resistance. In the case of discrepant results, we find that PSQ can be a valuable tool for rapidly evaluating LPA-indeterminate results instead of repeating phenotypic DST,

### Table 3 LPA mutation findings and distribution for molecular evaluation of rifampicin susceptibility

<table>
<thead>
<tr>
<th>LPA result</th>
<th>Mutation</th>
<th>Mutants</th>
<th>EPTB</th>
<th>PTB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mutant 1</td>
<td>D516V</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mutant 2A</td>
<td>H526Y</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mutant 2B</td>
<td>H526D</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mutant 3</td>
<td>S531L</td>
<td>144</td>
<td>18</td>
<td></td>
</tr>
</tbody>
</table>

LPA = line-probe assay; PTB = pulmonary tuberculosis; EPTB = extra-pulmonary TB.

### Table 4 LPA mutation findings and distribution for molecular evaluation of isoniazid susceptibility

<table>
<thead>
<tr>
<th>LPA result</th>
<th>Mutation</th>
<th>Mutants</th>
<th>EPTB</th>
<th>PTB</th>
</tr>
</thead>
<tbody>
<tr>
<td>(kat) G Mutant 1</td>
<td>AGC–315–ACC</td>
<td>153</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>(kat) G Mutant 2</td>
<td>AGC–315–ACA</td>
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<tr>
<td>(kat) G Mutant 1 and (inh) A Mutant 1</td>
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<tr>
<td>(inh) A Mutant 1</td>
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<td>0</td>
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<tr>
<td>(inh) A Mutant 2</td>
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<tr>
<td>(inh) A Mutant 3A</td>
<td>–8T/C</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>(inh) A Mutant 3B</td>
<td>–8T/A</td>
<td>3</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

LPA = line-probe assay; PTB = pulmonary tuberculosis; EPTB = extra-pulmonary TB.
as the technology appears to resolve discrepancies in line with phenotypic DST results.

Conflicts of interest: none declared.

References


CONTEXTE : Bien que des tests de sonde en ligne (LPA) soient prometteurs, peu de recherches ont été menées pour éclaircir la vraie nature des résultats indéterminés des LPA ou d’évaluer la performance de ces tests sur un large éventail d’échantillons cliniques.

OBJECTIF : Évaluer la performance des résultats du test GenoType® MTBDRplus LPA disponible dans le commerce contre celle de la culture par le test conventionnel BACTEC™ MGIT™ 960 et du test de pharmacorésistance (DST) sur 308 échantillons de TB pulmonaire (TBP) et 32 échantillons extrapulmonaires.

RÉSULTATS : Les résultats LPA invalides (définis comme la bande d’identification manquante de Mycobacterium tuberculosis) ont été obtenus pour 18 échantillons de TBP et exclus de l’analyse ultérieure. La sensibilité et la spécificité du test MTBDRplus pour la TB multirésistante, en se basant sur les résultats obtenus pour les 322 échantillons restants, ont été de respectivement 95,2% et 95,1%. De 290 échantillons TBP, 40 (13,7%) ont été indéterminés sur LPA (défini comme l’absence du type sauvage et des bandes de mutation correspondantes) pour l’isoniazide (INH) et/ou la rifampicine (RMP) et ont ensuite été évaluées par pyroséquençage (PSQ). Contrairement à l’interprétation du LPA standard, les sensibilités à l’INH et à la RMP ont été confirmées à la fois par DST et PSQ chez 7,5% (3/40) et 27,5% (11/40) des échantillons indéterminés, respectivement.

CONCLUSION : Quand les résultats du test LPA n’ont pas été interprétables, le PSQ s’est avéré une technique valable et rapide de résolution.

RESUMEN

MARCO DE REFERENCIA: Aunque los ensayos de hibridación en tiras en sondas (LPA) son prometedores, se ha hecho poca investigación para elucidar los resultados indeterminados LPA o evaluar la eficiencia de estos ensayos en una amplia gama de muestras clínicas.

OBJETIVO: Evaluar el rendimiento diagnóstico de la prueba comercial GenoType® MTBDRplus por LPA, en comparación con los resultados del enfoque clásico de cultivo en el sistema BACTEC™ MGIT™ 960 y las pruebas de sensibilidad a los medicamentos (DST), en muestras provenientes de 308 casos de tuberculosis pulmonar (TBP) y 32 casos de TB extrapulmonar.

RESULTADOS: En 18 muestras de casos de TBP se obtuvo un resultado indeterminado con la prueba LPA (definidos como la ausencia de la banda de identificación de Mycobacterium tuberculosis); estas muestras se excluyeron de los siguientes análisis. A partir de los resultados obtenidos con las 322 muestras restantes, la prueba MTBDRplus exhibió una sensibilidad de 95,2% y una especificidad de 95,1% en el diagnóstico de la TB multidrogorresistente. En el 13,7% de las muestras de casos de TBP (40/290) la prueba LPA produjo un resultado indeterminado para isoniazida (INH), rifampicina (RMP) o ambas (definido como la ausencia de ambas bandas, es decir de la cepa natural y también las bandas correspondientes a la mutación); estas muestras se evaluaron luego por pirosecuenciación (PSQ). En contraste con la interpretación habitual de resistencia frente a un resultado indeterminado con la prueba LPA, las pruebas corrientes de DST y la PSQ confirmaron la sensibilidad a INH en el 7,5% (3/40) y a RMP en el 27,5% (11/40) de las muestras indeterminadas.

CONCLUSIÓN: Cuando los resultados LPA no fueron interpretables, la PSQ resultó una técnica valiosa y rápida para resolver la incógnita.