A minimally instrumented method for the detection of rifampicin resistance-causing mutations in Mycobacterium tuberculosis utilizing lateral flow readout

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Keywords: Antimicrobial resistance, GeneXpert, line probe assay, global health, point of care diagnostics

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ABSTRACT

Genotypic methods for the determination of antimicrobial resistance in Mycobacterium tuberculosis (M.tb) require expensive instruments, which limits their availability in peripheral locations. We present a minimally instrumented method for the detection of the four most common mutations associated with rifampicin resistance in M.tb: S531L, H526Y, H526D, and D516V. The detection is based on the oligonucleotide ligation assay, coupled with lateral flow readout. The assay can detect wild-type and mutant DNA from as few as 10 and 100 gene copies per reaction, respectively. In heterozygous samples, the assay can detect < 3% mutant DNA for all 4 mutations. Preliminary validation of the assay was carried out using genomic DNA extracted from 29 M.tb isolates being cultured at the ICMR-National Institute for Research in Tuberculosis in Chennai, India. The assay achieved a sensitivity and specificity of 100% for the detection of M.tb, and 90.90% and 100% respectively, for the detection of rifampicin resistance. The assay is simple to extend to other resistance-causing mutations and may aid in the reduction of instrumentation associated with current TB genotypic AMR detection.

INTRODUCTION

Tuberculosis remains one of the most widespread diseases in the world. In 2022, TB caused 10.6 million infections and 1.3 million deaths globally, marking the second leading cause of deaths from a single infection after COVID-19[1]. Antimicrobial resistance (AMR) is a major deterrent in the path of TB eradication. The World Health Organization (WHO) estimates that in 2022, 0.41 million people developed rifampicin resistant (RR) TB or multi-drug resistant (MDR) TB i.e., resistant to both rifampicin and isoniazid. However, only 43% of them received treatment. The large gap between the estimated and diagnosed drug resistant-TB (DR-TB) cases highlights the lack of accessible diagnosis for DR-TB. In addition to MDR-TB, XDR-TB (extensively drug-resistant TB) is also emerging. XDR-TB is resistant to rifampicin (and may also be resistant to isoniazid), and resistant to at least one fluoroquinolone (levofloxacin or moxifloxacin), and to at least one other Group A drug (bedaquiline or linezolid).

AMR can be detected by phenotypic culture-based tests or genotypic nucleic acid amplification-based tests (NAATs). Although considered gold standard, phenotypic drug susceptibility testing (DST) for TB has long turnaround times of 6-8 weeks[2]. Genotypic DSTs are rapid and accurate and are recommended by WHO as the initial diagnostic tests for early detection of TB and DR-TB in suspected populations[1]. Drug resistance in TB is associated with well characterized point mutations in the DNA of the pathogen. In the current TB diagnostic workflow, mutations associated with rifampicin resistance are detected using instrumented tests like the Cepheid Xpert MTB/RIF or Truenat MTB-RIF Dx as frontline molecular tests. These automated instruments feature real time PCR amplification of the target nucleic acids and fluorescent probe-based readouts of mutations. A more thorough point mutation workup may be conducted, if needed, by using the line probe assay (LPA)[3]. This assay is based on the surface hybridization of PCR-amplified target DNA to a large number of mutation-specific

probes, followed by enzyme-mediated colorimetric signal generation. While the LPA can detect a large number of mutations, it involves an open-surface hybridization step performed in a specialized instrument. Current TB genotypic diagnostic methods are thus reliant on instruments that may prove to be very expensive for health systems in low- and middle-income countries[4]. Development of next-generation M.tb drug susceptibility tests that can be conducted at peripheral locations is an important WHO target product profile[5,6].

The first line antibiotics for TB are rifampicin, isoniazid, pyrazinamide, and ethambutol. Second line drugs – fluoroquinolones such as levofloxacin, moxifloxacin, and Group A drugs may be used when the infection is resistant to first line drugs[7]. The most used TB molecular tests like Xpert MTB/RIF and Truenat MTB-RIF Dx detect mutations in the 81 bp rifampicin resistance determining region (RRDR) of *rpoB* gene of M.tb. Around 96% of mutations conferring resistance to rifampicin are located in this 81bp region[8]. However, there is a need for tests that can detect resistance against other antibiotics, in addition to rifampicin. Molbio Diagnostics has developed the Truenat MTB-INH, a separate cartridge for the detection of resistance to isoniazid. Cepheid has developed the Xpert MTB/XDR test, which detects resistance to isoniazid, fluoroquinolones, and amikacin. However, to detect multiple resistances in a single cartridge, the test requires a more advanced 10-color fluorescence instrument, further increasing instrument cost.

With the goal of minimizing the instrumentation associated with TB genotypic AMR testing, in this article, we present a molecular assay for the detection of rifampicin resistance via multiple point mutations in the RRDR region of the rpoB gene of M.tb. The presented assay features the oligonucleotide ligation assay (OLA), coupled to lateral flow readout, and is based on the OLA-Simple technology developed by Panpradist et al.[9] for the detection of HIV-1 drug

resistance. The assay is designed to detect the four most common mutations in the RRDR located at codons 531(TCG531TTG), 526(CAC526TAC and CAC526GAC), and 516(GAC516GTC). The workflow consists of i) PCR amplification of target nucleic acids, ii) aliquoting of PCR products into multiple tubes for ligation-based point mutation detection (one tube per codon), and iii) lateral flow detection of the products of each ligation reaction. The entire workflow, starting from purified nucleic acids, only requires a thermal cycler (not a real-time PCR machine). Validation conducted using nucleic acids extracted from 29 M.tb isolates being cultured at the ICMR-National Institute of Research in Tuberculosis in Chennai, India, produced high sensitivity and specificity towards the detection of rifampicin resistance. The assay is easy to expand to more mutations and may aid in the development of minimally instrumented tests for comprehensive TB AMR profiling in peripheral locations.

EXPERIMENTAL SECTION

Plasmid templates: *Wild type plasmid:* A 550 bp sequence of the rpoB gene consisting of the 81 bp RRDR was inserted in a PUC 57 vector and used as the wild type template. *Mutant type plasmids:* Four mutant plasmids with a 225 bp sequence of the rpoB gene comprising the mutations of interests (531TTG, 526GAC, 526TAC, 516GTC) inserted in the TOPO vector were received as kind gifts from Dr. Haldar (PGIMER, Chandigarh, India) and used as mutant templates. The plasmids were transformed in DH5α competent cells. Details of bacterial transformation is provided in Supporting information (SI; Section 1). When needed, the plasmids were extracted from the transformed cells using a plasmid transformation kit (QIAprep® spin miniprep kit; 27104) and used as PCR templates after suitable dilution.

Polymerase chain reaction (PCR): A PCR assay was designed to amplify a 163 bp region of the rpoB gene of M.tb consisting of the 81 bp RRDR. A 20 µL PCR mixture consisted of 1×

PCR buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$), 250 µM of each dNTP, two units of Takara Taq DNA polymerase (Juniper Life Sciences; R001A), 0.4 µM each of forward and reverse primers, and 2 µL of plasmid template. The cycling conditions were as follows: initial denaturation at 95 °C for 5 min followed by 35 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 20 s, and a final extension at 72 °C for 7 min. PCR was conducted either in the Applied Biosciences Veriti™ Thermal Cycler or in the portable PCR instrument, KeyPCR by 30M Genomics Private Limited, Hyderabad, India. Amplified products were loaded on a 2% agarose gel and visualized using ethidium bromide staining post gel electrophoresis.

Oligonucleotide Ligation Assay (OLA): Products of the PCR reaction were aliquoted into multiple OLA reactions – one each for the detection of a single point mutation. Each OLA reaction consisted of 3 probes: a wild-type (WT), a mutant (MUT) and a common (COM) probe. The 531 and 526 codons have two mutations of interest, therefore the MUT probes for detecting 531 and 526 mutations consist of mixed base K, which consists of 50% dTTP and 50% dGTP. Sequences of the primers and probes are provided in the Supporting Information Table S1. A 20 µL OLA mixture consisted of 1X Taq DNA ligase buffer (20 mM Tris-HCl, 25 mM Potassium Acetate, 10 mM Magnesium Acetate, 1 mM NAD 1, 10 mM DTT, 0.1% Triton® X-100), 32 units of Taq DNA ligase (New England Biolabs Inc.; M0208S), 50 nM each of WT, MUT and COM probes (unless otherwise mentioned), and 1 µL of PCR amplicons. Cycling conditions were as follows: 40 cycles of 95°C for 30s and 47°C for 1min. These cycling conditions were optimized by visualization of the products of ligation using urea-TBE gels, which showed a band specific to the ligated product in the case of successful ligation (SI Section 2).

Lateral flow assay (LFA): LFAs were prepared for the detection of products of OLA reactions. The LFAs consisted of nitrocellulose membranes (NC FF120HP; Cytiva) attached to cellulose wicking pads (C083; Millipore), assembled over a self-adhesive backing card (Kenosha BV, Netherlands). Sample pads and conjugate pads were not used. The LFAs featured three lines – two test lines (WT and MUT) and one flow control line, dispensed using a lateral flow reagent dispenser (Claremont Bio Solutions, Upland, CA, USA). The WT test line was created by dispensing 1 mg/mL anti-FITC antibody (Abcam, Inc., Cambridge, MA, USA.; ab19224); the MUT line by dispensing 1 mg/mL anti-digoxigenin (DIG) antibody (Abcam, Inc., Cambridge, MA, USA.; ab64509); and the control line by dispensing 0.5 mg/mL BSA-Biotin (Sigma Aldrich; A8549). Strips of width 3 mm were cut using an automatic guillotine cutter. To visualise the ligated probes on LFA, 18 µL of OLA product was mixed with 2 µL of 6 µM competing oligomers and the mixture was subjected to four additional thermal cycling of OLA. After thermal cycling with competing oligomers, 2 µL of the OLA product was mixed with 0.8 µL of 10 OD streptavidin-gold conjugate (referred to as "gold conjugate" here onward; Abcam; ab186864) and 37 µL of running buffer (1X PBS, 0.05% Tween 20 and 1% BSA, pH 7.4) in a 96 well plate, and an LFA strip was dipped in the well. Images of the strips were acquired after 15 minutes.

Image analysis and calculation of the limit of detection

LFA strips were imaged on a Canon LiDE 220 flatbed scanner and the acquired images were analysed using ImageJ software. The image was split into red, green, and blue channels and the green channel was used for quantifying the test line intensity because it offered the maximum contrast. The method for measuring the test line intensity was as described by Sathishkumar et al.[10] In the case of heterozygous samples, the limit of detection (LOD) was calculated for the percentage of mutated product, using a method described by Armbruster et

al.[11] . According to the method, the LOD is calculated as $LOD = LOB + 1.645(\sigma_{low})$, where LOB is the limit of blank and σ_{low} is the standard deviation of test line signal obtained from a low concentration sample. The LOB is calculated as $LOB = \mu_{blank} + 1.645(\sigma_{blank})$, where μ_{blank} and σ_{blank} are the mean and standard deviation, respectively, of the test line signals obtained from analytical blanks.

RESULTS AND DISCUSSION

The molecular mechanism for the detection of point mutations

The overall workflow, starting from extracted nucleic acids, comprises of three steps: i) target DNA amplification using PCR, ii) aliquoting products of the PCR reaction into 3 ligation tubes – one each for the detection of mutations at the 516, 526, and 531 codons, and iii) lateral flow detection of the products of ligation (Fig. 1A). The molecular mechanism involved in genotyping is shown in Fig. 1B and relies on the high specificity of the DNA ligase enzyme to ligate, i.e. form a covalent bond in between two single DNA strands, only when they are bound to a target DNA strand with full complementarity. Each ligation assay consists of 3 end-labelled DNA probes – COM, WT, and MUT. The COM probe is biotinylated at the 3'end and phosphorylated at the 5' end. The WT and MUT probes are tagged with FITC and DIG, respectively, at their 5' ends.

The COM probe is designed to be fully complementary to the target, and its 5' end is located one nucleotide after the site of mutation (Fig. 1B). The WT and COM probes start upstream the mutation and their 3' ends overlap with the site of the mutation (Fig. 1B). The WT and MUT probes differ only by a single nucleotide at their 3' end; the WT probe is fully complementary to the WT target and the MUT probe is fully complementary to the MUT target. During the ligation step, all probes bind the target. However, depending on whether the target

is WT or MUT, only the corresponding WT or MUT probe binds the target with full complementarity, and only that probe can be ligated with the COM probe. Ligation results in the production of a longer DNA strand that is bi-labelled – biotinylated at the 3' end and tagged with FITC in the case of WT target or DIG in the case of MUT target (Fig. 1B). These bi-labelled products can subsequently be detected on LFAs.

The LFAs consist of 3 lines – two test lines and one control line (Fig. 1C). The first and second test lines contain anti-DIG and anti-FITC antibodies, respectively. In the case of MUT target, the DIG-tagged bi-labelled product binds to the first line, while in the case of WT target, the FITC-tagged bi-labelled product binds to the second line. The biotin on the other end of the products is used for visualization using streptavidin-gold conjugate. Thus, the presence or absence of the mutation is determined by which test line shows a signal. An important point to note is that the mismatched WT or MUT probe also binds the target but does not get ligated to the COM probe (Fig. 1B). Nonetheless, the complex thus formed is bi-labelled and produces a signal at the corresponding test line (SI Section S3). It is therefore critical to denature the product at the end of ligation to separate the un-ligated probes. Different physical and chemical denaturing agents were tested to eliminate the false signal (SI Section S3). The most effective strategy was the use of competing oligomers, as described by Panpradist et al.,[9] which involves displacing the ligation probes from the target by the addition of a high concentration of un-labelled oligomers that are complementary to the target. Overall, an important benefit of this genotyping scheme is that both the PCR amplification and ligation can be conducted in a thermal cycler (real time PCR is not needed). We have demonstrated that the scheme can be conducted using only a portable thermal cycler (keyPCR).

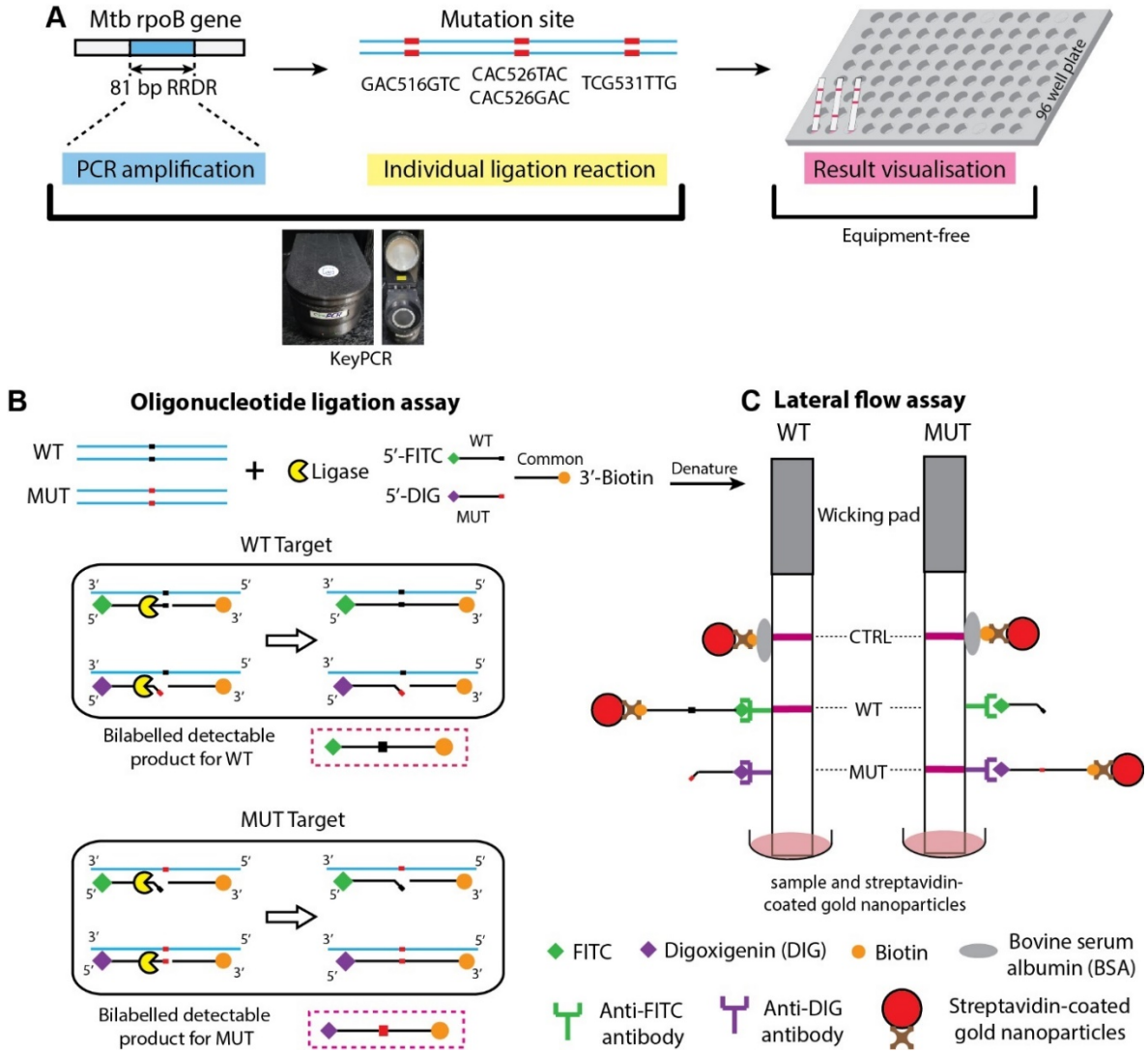

Figure 1. *The molecular mechanism for the detection of point mutations.* ***(A)*** *The three step procedure includes i) PCR amplification of the 81 bp RRDR, ii) individual ligation reaction on the amplified products to detect mutations at codons 516, 526, and 531, and iii) signal readout in a lateral flow dipstick format. The PCR and ligation reactions only require a portable thermal cycler.* ***(B)*** *The detection of each mutation utilizes 3 end-labelled probes. Based on the type of target, the ligase enzyme forms a covalent bond either between the WT and COM probe (for WT target) or between the MUT and COM probe (for MUT target) to form FITC-Biotin or DIG-Biotin bilabelled products, respectively.* ***(C)*** *Ligation products, post denaturation, are detected in lateral flow strips consisting of 3 lines – 2 test lines (WT and MUT) and one flow control line. The FITC-Biotin product is captured by anti-FITC antibodies at the WT test line and the DIG-Biotin product is captured by anti-DIG antibodies at the MUT test line.*

Visualization of ligated products using gel electrophoresis

For initial optimization of the ligation reactions, denaturing urea-TBE gel electrophoresis was used as the readout method, because this readout method did not involve end-modified probes, and therefore it was more economical to test multiple probe combinations. Denaturing gels are required for this purpose because the product of interest is single stranded ligated DNA product, which could coil up in unpredictable shapes and would not be visible as a sharp band without denaturation. Urea-TBE gels were prepared as described in SI Section 2. To visualize the ligated product on the gel, the assay for the detection of a mutation at the 531 codon was used. The PCR amplicons were spiked into the ligation reaction. A 44 nt synthetic strand having the same sequence as the intended ligated product was used as the positive control (P; lane 8; Fig. 2A). When conditions were appropriate for ligation, a band was observed at the same location (+; lane 4; Fig. 2A), indicating successful ligation. When the same reaction was conducted without ligase enzyme (NL; lane 2; Fig. 2A) or without using a phosphorylated common probe (NP; lane 3; Fig. 2A), the band at that position disappeared, indicating that ligation was not successful under these conditions, as expected. Note that another band appears very close to, and just below the location of the ligated band (*; lanes 2-4; Fig. 2A) and is attributed to primer dimers that are carried over from the PCR reaction. To confirm that this additional band was because of primer dimers, PCR products were analyzed using agarose gel electrophoresis, and the product band was cut and gel purified. When this product was used as the template for ligation, the extra band disappeared (lanes 5-7; Fig. 2A). The same three conditions for ligation were repeated using gel-purified amplicons as the target. Again, the no ligase condition (NL; lane 5; Fig. 2A) and non-phosphorylated probe (NP; lane 6; Fig. 2A) did not produce the ligated product, while the full reaction (+; lane 7; Fig. 2A) produced the ligated product.

The specificity of ligation toward the presence or absence of a mutation is demonstrated next. Gel purified product was used for this experiment to prevent the appearance of the primer dimer band. Three reactions were conducted using the WT template (lanes 2-4; Fig. 2B) and three were conducted using the MUT template (lanes 5-7; Fig. 2B). The three conditions involved the use of different probe combinations – **WC**: WT and COM probe only; **MC**: MUT and COM probe only; and +: all three probes WT, MUT, and COM. For both WT and MUT target, ligated product was produced when all 3 probes were used (+; lanes 4 and 7; Fig. 2B). However, for the WT target, only the WC combination and not the MC combination produced a ligated band (lane 2; Fig. 2B). Analogously, for the MUT target, only the MC combination produced a ligated band (lane 6; Fig. 2B). This result confirms the specificity of the ligase enzyme to differentiate a single nucleotide mismatch between the target and the probe.

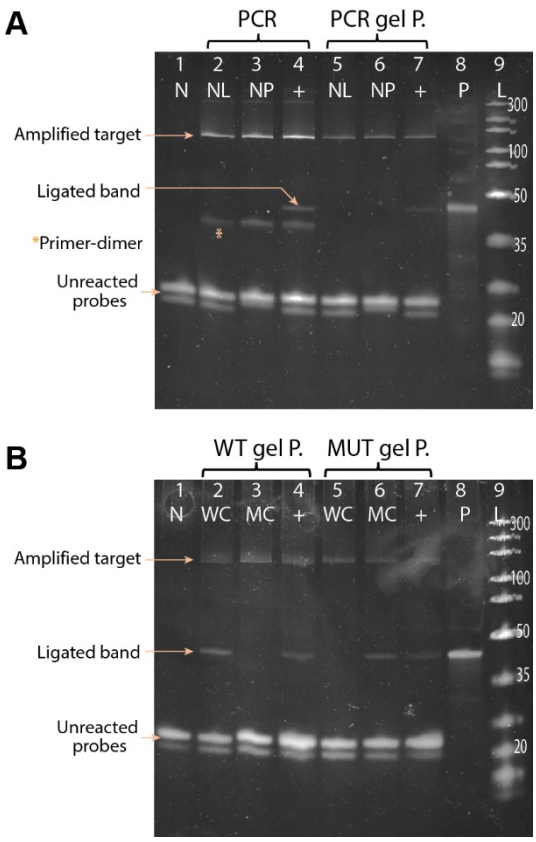

Figure 2. *Visualization of ligated products using gel electrophoresis.* ***(A)*** *Lane 1: No template control; Lanes 2-4: no ligase control (NL); not phosphorylated common probe*

control (NP); and all ligation components (+), using direct PCR products as templates for ligation. Lanes 5-7: similar conditions as lanes 2-4 but using gel purified PCR amplicons. Lane 8: Reference 44-mer to mark the location of the ligated product. The ligated band appeared only when all ligation components were present (+). The primer dimer bands (*) disappeared when using gel-purified amplicons. ***(B)*** *Investigation of the specificity of ligation reactions. Lane 1: No template control. Lanes 2-4: WT template with a mixture of WT and COM probe (WC); of MUT and COM probe (MC); and of MUT, WT, and COM probes (+). Lanes 5-7: Similar to Lanes 2-4 but using MUT template. Lane 8: Reference 44-mer to mark the location of the ligated product. For WT target, the ligated band was observed only if the mixture contained WT and COM probes (WC) and for MUT target, only if the mixture contained MUT and COM probes (MC).*

Detection of ligated products using lateral flow readout

Homozygous samples: After optimization of ligation conditions, lateral flow readouts for the ligated products were developed. The entire workflow was conducted for the detection of each mutation starting from 1000 copies of either WT plasmids or mutant plasmids (531L, 526Y, 526D, 516V). All targets were homozygous in this case, i.e. either 100% WT or 100% MUT. Results from 3 replicates for each condition are shown in Fig. 3. For the 531 codon, WT target produced signals only at the WT line, while the MUT target produced signals only at the MUT line of the LFA (Fig. 3A). For the 526 codon, two mutations are known to exist – 526D and 526Y, both of which could be detected by using a 1:1 mixed population of MUT probes (Fig. 3B). Similarly, for the 516 codon, WT and MUT produced signals exclusively at the corresponding WT and MUT lines on the LFA (Fig. 3C). Thus clear digital distinction between the presence and absence of mutations is observed in the case of homozygous samples.

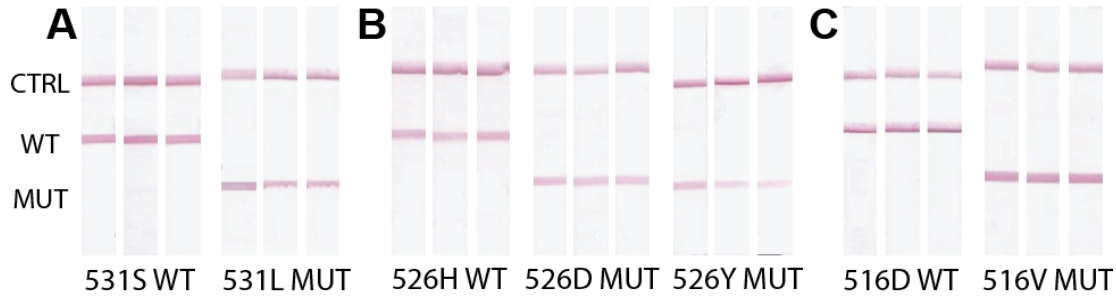

Figure 3. *Lateral flow detection of products of ligation for homozygous samples.* ***(A)-(C):*** *The OLA assay was performed on 100% WT target or 100% MUT targets containing the*

*531L mutation **(A)**, the 526Y and 526D mutations **(B)**, and the 516V mutation **(C)** (N=3 for all cases). The assay demonstrated a clear distinction between WT and MUT targets.*

Heterozygous samples: The entire workflow was subsequently performed on samples containing a mixture of WT and MUT targets. Mixtures containing different percentage of WT and MUT plasmids were prepared, maintaining the total number of target plasmid copies at 1000. The template mixture was amplified using PCR, followed by ligation and LFA detection for each mutation. The intensities of WT and MUT lines changed with increasing fraction of MUT target. For example, for detection of the 531L mutation, only the WT line was visible for a sample containing 0% MUT (Fig. 4A; left end) while only the MUT line was visible for 100% MUT (Fig. 4A; right end). In between, as the fraction of MUT target increased, the intensity of the MUT test line increased. The ratio of the signal obtained at the MUT test line to WT test line was plotted against %MUT and was found to increase monotonously (Fig. 4B). Similar behavior was observed for increasing fractions of the 526D mutation (Fig. 4C,D) and 516V mutation (Fig. 4E,F). This demonstrates the ability of the method to determine the fraction of mutant targets in a mixed sample. The LOD for MUT targets was found to be 2.5% for S531L, 2.14% for H526D, and 2.96% for D516V. The LODs of genotypic hetero resistance for the three mutations of interest ranges from 20% to 70% for GeneXpert, 5% to 10% for LPA-HAIN, and 1% to 10% for LPA-Nipro[12], indicating comparable or superior LODs of our method compared with the current gold standard methods.

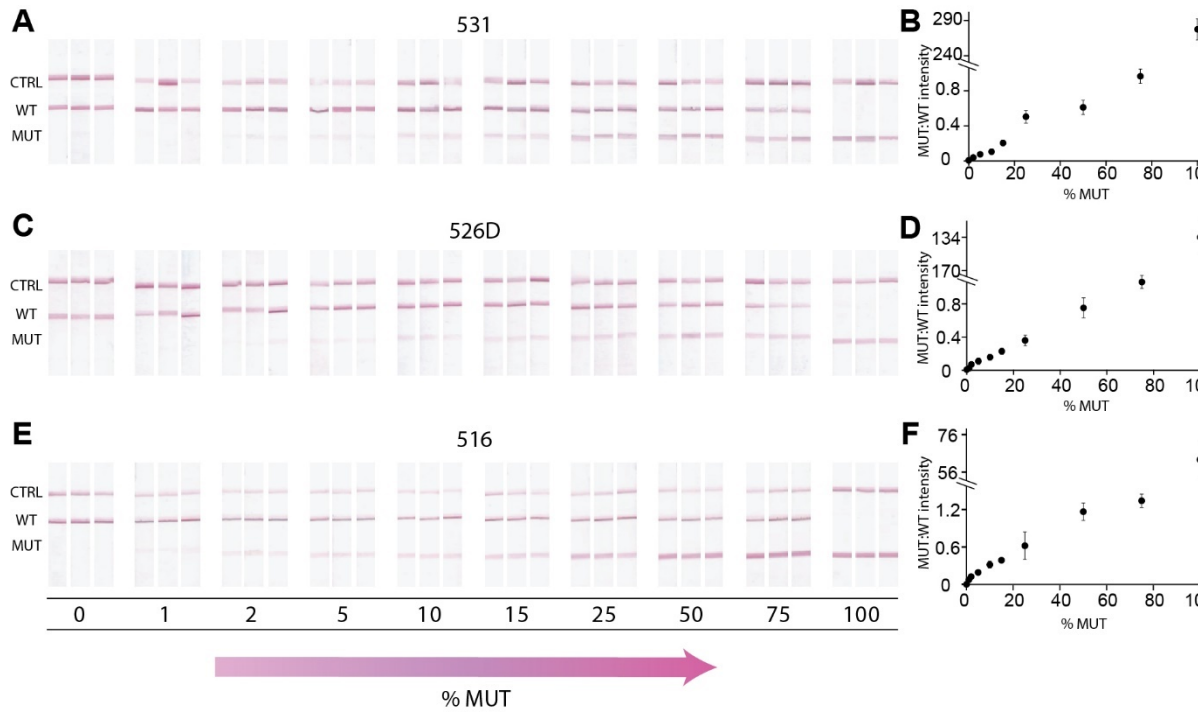

Figure 4. *Lateral flow detection of products of ligation for heterozygous samples. Samples containing increasing fractions of MUT target were subjected to the entire OLA workflow for the 531L mutation* ***(A-B)****; 526D mutation* ***(C-D)****; and the 516V mutation* ***(E-F)****. In each case, the ratio of MUT:WT test line intensities increased monotonously with increasing fraction of MUT target DNA* ***(B)****,* ***(D)****,* ***(F)****.*

The limit of detection for Mycobacterium tuberculosis

It is important to detect WT and MUT targets from as few target DNA copies as possible. Because the above experiments were conducted starting with 1000 target copies, it was important to figure out the lower limit of copies which may be detected using this method. This was investigated using WT and S531L mutant plasmids. Different concentrations of target DNA were prepared by conducting 10X serial dilutions of a solution containing 10^4 copies/µL of WT and S531L MUT plasmid. The targets were PCR amplified and subjected to ligation followed by LFA. Visible signals were obtained for both WT and MUT targets for 10^4, 10^3, and 10^2 copies per PCR reaction (Fig. 5A). However, for 10 copies per reaction, only the WT target could be detected (Fig. 5A). The no-template control (NTC), which did not contain any target DNA, did not show signals at either test line, as expected (Fig. 5A). Thus, the limit of detection for the number of target copies may be assumed to be 100 copies or lower. The loss

of signal for MUT targets at 10 copies is likely because of less efficient binding between DIG and anti-DIG antibodies compared to FITC and anti-FITC antibodies. This issue may potentially be overcome by increasing the concentration of anti-DIG antibody at the MUT line.

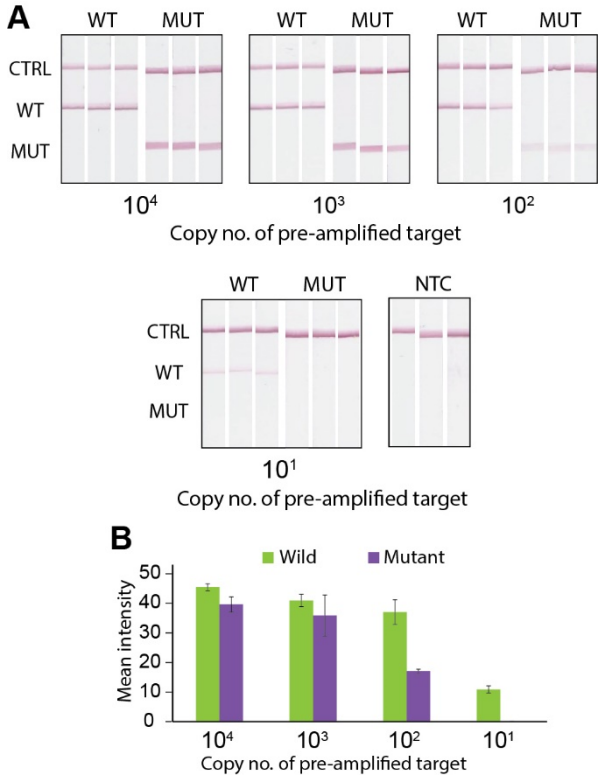

Figure 5. *Analytical sensitivity of the assay.* ***(A)*** *LFA results for different starting copy numbers of WT and MUT target.* ***(B)*** *Mean test line intensity vs starting copy number for WT and MUT targets. The assay could detect 10 copies of WT plasmid and 100 copies of 531 MUT plasmid. NTC: no template control, n=3*

Validation using bacterial isolates

A proof of concept of the molecular test was performed by testing genomic DNA extracted from 29 M.tb isolates being cultured at the National Institute of Research in Tuberculosis (NIRT), Chennai, India. Reagents were carried from the Indian Institute of Science (IISc), where the test was developed, to NIRT in Chennai, and the 29 samples were tested using the full workflow at NIRT. The 29 samples were previously sequenced using whole genome sequencing at NIRT and provided blinded to IISc for testing with the newly developed method.

All 29 samples were PCR amplified, and for each sample, 1μL of the amplified DNA was aliquoted into three ligation reactions, designed for the detection of mutations at 531, 526, and 516 codons. Products of ligation were then introduced into LFAs. Results were thus read using 3 LFA strips per sample.

The following rules were used to interpret test results:

1. A sample was considered TB-negative if none of the three strips produced test line signals – neither WT nor MUT.
2. A sample was considered TB-positive if all three strips produced test line signals, either WT or MUT.
3. The test was considered to have failed if test line signals, either WT or MUT, were produced only on 1 or 2 strips.
4. A sample was considered rifampicin-resistant if one or more of the strips produced a MUT test line signal. In such a case, the strip that generated the MUT test line signal indicated the location of the mutant codon.

   A summary of test results obtained using the above rules is presented in Table 1. Out of the 29 samples tested, 4 samples (13.8%) were reported as failed tests as they failed to produce test line signals in all three strips. Out of the remaining 25 samples, 8 samples were reported as TB-negative and 17 as TB-positive. All samples were correctly identified as TB-positive or -negative (compared to the reference standard), resulting in 100% sensitivity and specificity of TB detection. Out of the 17 TB-positive samples, the test reported 7 samples as WT/rifampicin-sensitive and 10 as MUT/rifampicin-resistant. According to the reference standard, 6 samples were WT/rifampicin-sensitive and 11 were MUT/rifampicin-resistant. The test thus misidentified one rifampicin-resistant sample as rifampicin-sensitive. Overall, it

produced a sensitivity and specificity of 90.9% and 100%, respectively for the detection of rifampicin-resistance. Out of the 11 rifampicin-resistant TB-positive samples, the test correctly reported 8 samples as having a mutation at codon 531, and 2 samples as having a mutation at codon 526. For one rifampicin-resistant sample, the test failed to detect a mutation at codon 526. Images of all lateral flow strips for these experiments are provided in SI Section 5.

Table 1: Comparison of OLA-LFA assay results against WGS

Sample No.	WGS		Developed assay	
	TB/DR-TB status	Mutant codon	TB/DR-TB status	Mutant codon
1	-/-	NA	-/-	NA
2	-/-	NA	-/-	NA
3	-/-	NA	-/-	NA
4	-/-	NA	-/-	NA
5	-/-	NA	-/-	NA
6	+/-	-	+/-	-
7	+/-	-	+/-	-
8	+/-	-	+/-	-
9	-/-	NA	-/-	NA
10	-/-	NA	-/-	NA
11	-/-	NA	-/-	NA
12	+/+	531	+/+	531
13	+/+	531	+/+	531
14	+/+	531	+/+	531
15	+/+	526	+/+	526
16	+/-	-	+/-	-
17	+/+	516	FAILED TEST	
18	+/+	531	+/+	531
19	+/+	531	+/+	531
20	+/+	531	FAILED TEST	
21	+/+	526	+/+	526
22	+/+	526	+/-	-
23	+/+	531	+/+	531
24	+/-	-	+/-	-
25	+/-	-	FAILED TEST	
26	+/-	-	FAILED TEST	
27	+/+	531	+/+	531
28	+/+	531	+/+	531
29	+/-	-	+/-	-

Overall, we have described a minimally instrumented assay for detecting the key point mutations that confer rifampicin resistance to M.tb. This is the first report that couples OLA and LFA for M.tb point mutation detection. The assay accomplishes good limits of detection for M.tb (10 copies/µL and 100 copies/µL for WT and MUT target, respectively) and, in heterozygous samples, limits of detection for mutant fractions that are comparable to or surpass those of GeneXpert and LPA. Compared to instruments like Truenat and GeneXpert, the OLA-LFA workflow is amenable to the incorporation of a larger number of mutations to be detected, because it is not limited by the number of fluorescent channels. Further, the LFA strip used for detection is universal, i.e. the same type of strip can be used for the detection of all mutations. The incorporation of an additional mutation detection in the workflow is thus straightforward and can be accomplished simply by the addition of an additional ligation reaction for the desired mutation. The detection of mutations in other genes may be accomplished by incorporating multiplex PCR in the workflow to amplify multiple genes of interest.

Despite the good performance and desirable characteristics of the test, it is important to acknowledge certain gaps/drawbacks of the approach. First and foremost, extraction of nucleic acids from complex samples has not been incorporated in this workflow. Nucleic acid extraction will add to the number of user steps in the test and may require additional instruments. Further, while conducting tests on 29 blinded samples, it became apparent that the test workflow is manually intensive and would require a moderately-trained user to conduct a number of timed steps. There is thus a tradeoff between the number of user steps and complexity of instrumentation required to conduct a diagnostic test. The currently developed test minimizes instrumentation but requires substantial user intervention. Additionally, opening PCR tubes to manually transfer PCR products into ligation tubes makes the test susceptible to amplicon contamination. However, such transfer of PCR products is routinely conducted in the

well-established LPA genotypic assay. Potential solutions to overcome some of these shortcomings include microfluidic automation of the test workflow, or the one-pot integration of PCR and OLA, which are areas of current research in our group. Finally, it must be acknowledged that during the PCR primer design phase of this work, we failed to realize that large sequences of the RRDR region of M.tb are conserved in non-tuberculous mycobacteria (NTM). The current test may therefore falsely report NTM samples as M.tb-positive. However, this shortcoming may be overcome by a simple PCR primer redesign exercise, not affecting the design of OLA probes.

CONCLUSION

The work herein describes a molecular workflow for the detection of multiple drug resistance-conferring point mutations in M.tb, utilizing minimal instrumentation. The entire workflow, starting from extracted nucleic acids, may be conducted using only a portable thermal cycler (not a real time PCR machine). Currently, the most widely performed tests for genotypic AMR detection in M.tb are conducted in automated instruments like GeneXpert and Truenat, and their high cost leads to a substantial financial burden on health systems. This work presents a minimally instrumented method for genotypic AMR detection that may be conducted by moderately trained technicians, with the hope of accomplishing substantial reduction in capital costs to the health system, at the moderate cost of training manpower to conduct these tests. In low- and middle-income countries, where labor costs are relatively low, this may provide a feasible model to increase the penetration of genotypic AMR testing.

ASSOCIATED CONTENT

Supporting Information. The following Supporting Information is available free of charge on the ACS Publications website:

Bacterial transformation of plasmids; Sequences of oligomers; Urea-TBE gel; Cycle condition for OLA; Denaturation of OLA products; Optimizing concentration of probes for LFA; Clinical study data

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Author Contributions

A.C: Methodology, Investigation, Formal analysis, Writing – original draft; S.S: Resources, Supervision; B.J.T: Conceptualization, Methodology, Writing – review and editing, Supervision, Funding acquisition.

Notes

The authors declare no conflicts of interest.

Acknowledgements

This work was supported by a research grant from the Issac Center for Public Health (ICPH) at the Indian Institute of Science; a core research grant (CRG/2021/003397) from the Science and Engineering Research Board, India; a special COVID-19 consortium grant (BT/PR40322/COD/139/10/2020) and an extramural research grant (BT/PR50679/MED/32/998/2023) from the Department of Biotechnology, India, and a research grant awarded by Syngene International Limited. We sincerely thank Dr. Sagarika Haldar (PGIMER Chandigarh, India) for providing mutant plasmids (531L, 526D, 526Y, 516V) containing the RRDR, Dr. Ketan Ganar for synthesizing wild type plasmid, Mr. Venkat

Subramanian Ramesan and Mr. Raghuvaran for providing support during conduct of the OLA-LFA tests at ICMR-NIRT Chennai, India.

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