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Original Article

Enhancement of *M. tuberculosis* Line Probe Assay Sensitivity through Whole Genome Amplification of Low-Quantity DNA Released from Sputum and Archived on Chemically-Coated Cellulose Matrix Using an Isothermal Enzymatic Strand-Displacement Process

Krishna H. Goyani[1], Chirajyoti Deb[2], Daisy Patel[1], Shalin Vaniawala[3], Pratap N. Mukhopadhyaya[1*]

[1]*Wobble Base Bioresearch Private Limited, Pune, India.*
[2]*Arnold Palmer Hospital for Children, Orlando Health, 92 Miller Street, Orlando, FL, USA.*
[3]*New Civil Hospital, Jay Prakash Narayan Marg, Surat.*

[*]*Corresponding Author : pnm@wobblebase.in*

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Abstract *- In this study, thirty-nine sputum samples from tuberculosis (TB)-positive patients undergoing first-line therapy were collected and archived on a chemically coated cellulose matrix. DNA was extracted from these matrices and tested for Mycobacterium tuberculosis using the Xpert MTB/RIF Ultra assay. Seven samples tested positive for M. tuberculosis, with low levels of detection. End-point PCR yielded faint signals in four samples but no signal in the remaining three. A Line Probe Assay (LPA) detected pathogenic DNA in only one of the three PCR-negative samples. Remarkably, LPA-negative samples were successfully detected by LPA and end-point PCR following isothermal strand displacement-based whole genome amplification (WGA) of the stock DNA. The drug sensitivity profiles of these samples were consistent with the Xpert MTB/RIF Ultra results obtained using the original stock DNA. Additionally, sputum DNA from healthy individuals spiked with 125 ng, but not 1.25 ng, of M. tuberculosis culture DNA was detected by LPA. Following WGA, the 1.25 ng sample was also detected successfully by LPA, and its drug sensitivity profile matched that of the 125-ng sample. These findings indicate that WGA of sputum DNA from a cellulose matrix, even with a low pathogen load, can enhance the detection capabilities of LPA by enriching the genome target through an isothermal enzymatic strand displacement method. This method is promising for improving the diagnostic sensitivity of TB detection.*

Keywords *- Cellulose matrix, Whole Genome Amplification, Line Probe Assay, Xpert, End point PCR, Tuberculosis*.

1. Introduction

Tuberculosis is a serious infectious disease with worrisome statistics. It causes around 10.4 million cases and 1.8 million events of mortality in a year across the globe. It is estimated that approximately 4.3 million infected persons fail to be diagnosed [1]. The complexity of these disease compounds due to the rapid emergence of multidrug and extensively drug-resistant tuberculosis (MDR-TB and XDR-TB, respectively) poses a major challenge to efforts to control TB worldwide [2].

Culture-based methods for testing the drug susceptibility of M. tuberculosis using solid media are effective and reliable. However, one of the major shortcomings of this method is its long turnaround time, which may be between 8 and 12 weeks [2]. A relatively faster and more sophisticated alternative is the liquid-based culture method, which may take a long turnaround time of 4 to 6 weeks [3]. This long period of time to generate results is detrimental since it translates to long periods of nil or ineffective therapy, which promotes rampant transmission of the pathogen. This is the reason for the rapid evolution of molecular techniques for detecting *M. tuberculosis* and its drug-resistant variants [4]. The turnaround time here is often less than a day, and these methods generate reliable results.

Recently, line probe assays or LPAs have emerged as reliable and rapid molecular methods for the detection of *M. tuberculosis*, particularly their drug-resistant variants. Despite the complexity associated with the protocol, they reliably detect isoniazid (INH) and rifampicin (RIF) resistance in *M. tuberculosis*. They are comparatively less expensive than

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Xpert MTB assays (Cepheid, Sunnyvale, CA, USA). Furthermore, this test concluded with an average mean time of approximately 8 hours [5].

In line probe assay, the DNA from a clinical sample is extracted using conventional but rapid methods. The species-specific and drug-resistant determining regions within the *M. tuberculosis* genome are amplified with oligonucleotide primers tagged with biotin (Biotinylated probes). The labelled PCR amplicons were then hybridized to specific probes and immobilized on a solid support in the form of thin strips. These trapped hybrids were then detected by colourimetry using the classical biotin–avidin alkaline phosphatase detection protocol. The final result is in the form of thin colored lines that are scorable to the naked eye.

The line probe assay from Hain Life Science contains clinically useful variants. The GenoType MTBDR plus kit detects *the M. tuberculosis* complex as well as its resistance to rifampicin and/or isoniazid from clinical specimens and cultures, whereas the GenoType MTB DRsl kit was used to detect resistance to fluoroquinolones, aminoglycosides/cyclic peptides, and/or ethambutol from *M. tuberculosis* clinical specimens and cultures (Hain Lifescience GmbH, Nehren, Germany).

However, one of the shortcomings of the line probe assay is its relatively low level of sensitivity compared to other molecular assays in its league, such as Xpert MTB Rif Ultra (Sunnyvale, CA, USA). Studies indicate that the limit of detection of LPA is approximately 10,000 CFU/ml [6], which is similar to that of smear microscopy. In comparison, the culture-based method of detection of drug sensitivity in *M tuberculosis* has a low of 10-100 CFU/ml [7]. The LPA is likely prone to missing samples with a low bacillary load. This is particularly visible when patients are on first-line anti-tuberculosis treatment during the collection of samples, which often affects the quantity of target DNA in clinical samples. [8]

In recent years, techniques and approaches have evolved for superior methods of collecting, transporting, storing, and releasing DNA from *M. tuberculosis*-infected clinical samples, with special reference to sputum. Goyani et al. (2023)[4],[5] described a chemically coated cellulose matrix that could be effectively used for sputum storage and DNA-release devices with the advantage of optimal biocontainment properties [9]. The device demonstrated compatibility with cartridge-based nucleic acid amplification test platforms, such as Xpert MTB Rif Ultra and/or its variants (Sunnyvale, CA, USA) [10], which are known for their high level of sensitivity and specificity [11].

However, when reagent-coated matrixes such as those cited above are used for archival and release DNA from sputum containing a low quantum of infected *M. tuberculosis* bacilli, the low quantity of DNA results in false-negative results in line probe assays. In this study, we demonstrate the use of whole genome amplification of a heterogeneous DNA population released from human sputum DNA archived in a reagent-coated cellulose matrix [9] to convert false negative calls for low-target DNA-containing samples to true positive ones by artificially increasing the quantum of available DNA for line probe assays to detect *M. tuberculosis* drug resistance [12].

1.1. Research Gap and Problem Statement

Even with the progress in molecular diagnostic technologies like LPAs and cartridge-based nucleic acid amplification tests (NAATs), detecting *M. tuberculosis* remains challenging, especially in samples with low bacillary counts. LPAs are known for their quick and cost-efficient detection of drug-resistant TB. However, their relatively high detection threshold (~10,000 CFU/ml) limits their effectiveness in minimal bacterial DNA, such as in patients receiving treatment or those with paucibacillary TB. Similarly, although reagent-coated cellulose matrices have enhanced DNA preservation and biocontainment, their effectiveness diminishes when handling sputum samples with low bacterial content, often resulting in false-negative outcomes.

This limitation underscores the urgent need for a method that boosts the sensitivity of LPAs without significantly changing current workflows. In this research, we tackle this issue by using Whole Genome Amplification (WGA) to artificially increase the DNA quantity from sputum samples stored in a reagent-coated cellulose matrix. Our method seeks to enhance LPA performance in identifying drug-resistant TB in samples with low target DNA, thereby reducing the likelihood of false-negative results and improving diagnostic precision.

1.2. Novelty of the Work

This research introduces an innovative method to improve tuberculosis (TB) detection by combining Whole Genome Amplification (WGA) with a chemically coated cellulose matrix for sputum DNA storage. Unlike traditional liquid storage techniques, this cellulose matrix allows for stable DNA preservation and extraction, facilitating subsequent molecular analysis. The use of isothermal strand displacement-based WGA notably enhanced the sensitivity of the Line Probe Assay (LPA), allowing for the identification of Mycobacterium tuberculosis in samples that were initially negative due to low pathogen levels.

This improvement was confirmed through various molecular techniques, such as Xpert MTB/RIF Ultra and end-point PCR, demonstrating the wide-ranging applicability of WGA in TB diagnostics. Crucially, the drug resistance profiles of the amplified DNA remained unchanged from the original samples, indicating that WGA does not introduce bias in detecting genotypic resistance. These results suggest that

WGA-based DNA enrichment can boost the diagnostic sensitivity of TB detection from sputum samples, especially in cases where low bacterial load poses a challenge for standard molecular tests.

1.3. Current Methods of Pre-Amplification PCR

Whole Genome Amplification (WGA) methods have been devised to solve the problem of amplifying small DNA samples before PCR detection. Among these, multiple displacement amplification (MDA) is a widely adopted technique for single-cell WGA, utilizing Phi29 DNA polymerase and random primers [26]. However, MDA can lead to uneven genome coverage due to amplification bias and contamination issues. To mitigate these challenges, several advanced WGA techniques have been introduced. The droplet MDA method addresses amplification bias and contamination by confining WGA reactions within picoliter-sized droplets, thereby improving genome recovery from 59% to 89% in single Escherichia coli cells [26]. Another innovative approach is the improved degenerate oligonucleotide-primed PCR (iDOP-PCR), which employs a new thermostable DNA polymerase with strong strand-displacement activity and an enhanced primer design, resulting in superior quality amplified DNA libraries compared to traditional DOP-PCR and PicoPlex methods [25].

In conclusion, recent advancements in WGA techniques have significantly enhanced the amplification of low-quantity DNA templates. These methods offer increased specificity, amplification yield, and genome coverage while reducing bias and contamination. The choice of WGA technique depends on the specific application and the nature of the sample, with each method having its own strengths and limitations. As far as we know, no prior research has explored using BstEI-mediated isothermal amplification for the Whole Genome Amplification (WGA) of *Mycobacterium Tuberculosis* (MTB) DNA to enhance assay sensitivity. Conventional WGA methods, such as multiple displacement amplification (MDA) using Phi29 polymerase, are widely employed for whole genome amplification in various contexts. However, the potential role of BstEI, a thermostable restriction enzyme, in facilitating DNA fragmentation and subsequent amplification has not been examined in the realm of MTB detection. Our approach utilizes BstEI digestion to generate DNA fragments of an ideal size, which may improve amplification efficiency and boost the sensitivity of subsequent PCR-based assays. This novel technique could provide advantages over traditional methods, particularly when low-burden MTB samples require highly efficient DNA amplification for precise detection.

2. Materials and Methods

2.1. Clinical Sample of M. Tuberculosis

Human sputum samples infected with M. tuberculosis were obtained and spotted onto a reagent-coated cellulose matrix [9] at the Microcare Laboratory and Tuberculosis Research Centre, Gujarat, India. All samples were confirmed to be M. tuberculosis-positive using Xpert MTB/Rif assay (Sunnyvale, CA, USA).

2.2. Release of DNA for Line Probe Assay (LPA)

Reagent-coated cellulose matrix spotted with clinical samples returned as “Low” positive by Xpert MTB Rif assay were processed after incubation at room temperature for 48 hours to release DNA by soaking the membrane in sterile double distilled water for 15 minutes. Then, 650 µL of this solution was placed in a sterile, 2 ml centrifuge tube and mixed with 65 µL of 3 Molar sodium acetate (Sigma-Aldrich, St.Louis, MO, USA). Louis, MO, USA) followed by its precipitation by adding 1.4 ml of ice-cold 100% Ethyl alcohol. The mixture was centrifuged at 4^0C for 5 min to precipitate the DNA. The pellet was washed with 70% ethyl alcohol, air-dried, and suspended in 50 µL of sterile nuclease-free water. Five microliters of this DNA solution were used for the line probe and end-point PCR assays.

2.3. Processing of DNA for Line Probe Assay (LPA)

The Line Probe Assay was performed according to the manufacturer’s protocol (Hain Lifescience GmbH, Nehren, Germany). Briefly, all reagents were thawed on ice, and the PCR reaction mixture was prepared according to the manufacturer’s instructions. The precipitated DNA (5 µL) was added to the PCR mixture and placed on a thermal cycler to run the designated PCR program. For hybridization and detection, the PCR products were denatured by heating, quickly transferred to an ice-cold hybridization buffer, and added to the test strips placed in the hybridization tray. It was then incubated in a water bath at the temperature prescribed by the manufacturer and subjected to stringent washes to remove nonspecific DNA molecules. The processed strips were then incubated with conjugate reagent, washed, and re-incubated in substrate solution to visualize the results. A final wash was performed to preserve signals. The strips were then dried, and the results were interpreted by comparison with the reference guide provided in the kit.

2.4. End Point PCR amplification of precipitated DNA

For end-point PCR, 5 µL of precipitated DNA was used. Ten picomoles of forward and reverse primers [CP089781.1: g. 1470368 to g.1470387 (+) and CP089781.1: g.1470567 to g.1470588 (-), respectively] were added to a commercial Thermo Scientific PCR Master Mix (Thermo Fisher Scientific, USA) diluted to 1 × concentration with nuclease-free water. Target DNA was amplified in a total volume of 25 µL using a Perkin Elmer Applied Biosystems GeneAmp PCR System 9600. The thermal cycling conditions were as follows: 94^0C for 2 min (hold), 94^0C for 30 seconds, 58^0C for 30 seconds, 72^0C for 30 s (35 cycles), 72^0C for 2 min (hold) and 4^0C for 5 min (hold). Five µL of the PCR products were resolved on a 2% agarose gel (Seakem agarose, Lonza, Basel,

Switzerland) and stained with ethidium bromide (Sigma-Aldrich, St. Louis, MO, USA). Louis, MO, USA) at a final concentration of 0.5 µg/ml and the bands were recorded using a Canon EOS Rebel T7i digital camera with an orange filter. The gel images were analyzed using GelAnalyzer 23.1.1 software (www.gelanalyzer.com).

2.5. Formulating Dilutions of M. Tuberculosis DNA-Spiked Clinical Samples

M. tuberculosis DNA was extracted from cultures grown in Middlebrook 7H10 medium [13] using a QIAamp DNA mini kit (Qiagen, Hilden, Germany). The extracted DNA was added to *M. tuberculosis*-negative sputum DNA, and two different dilutions of the extracted DNA were prepared. In Dilution-1 stock DNA, the concentration of *M. tuberculosis* DNA was 25 ng/µL, whereas in Dilution-2 stock DNA, it was 0.25 ng/µL respectively.

Five microliters of these dilutions, computed to a total of 125 ng and 1.25 ng, respectively, were used for setting up the LPA or whole genome amplification reactions. All experiments were performed in triplicate to confirm the repeatability of the results unless otherwise mentioned.

2.6. Whole Genome Amplification (WGA) of the Cellulose Matrix Extracted Sputum DNA and M. Tuberculosis DNA-Spiked Clinical Samples

For whole genome amplification of the heterogeneous DNA released from the reagent-coated cellulose matrix spotted with *M. tuberculosis-positive* sputum or the sample (Dilution-2) formulated by spiking *M. tuberculosis* culture DNA with *M. tuberculosis*-negative sputum DNA, 5 µL of the DNA was used in a 25 µL reaction volume. The reaction composed of Tris-HCl (pH 8.8): 40 mM; KCl: 20 mM; $(NH_4)_2SO_4$:20 mM; $MgSO_4$:6 mM; dNTPs: 1.4 mM each; *Bst* DNA Polymerase (New England Biolabs (NEB): 16 units; Random primer: 5 µM (Thermo Fisher Scientific (Catalogue No. 48190011) and Betaine: 1 M final concentration. The incubation was performed on a Perkin Elmer Applied Biosystems Gene Amp PCR System 9600, and the incubation temperature was as follows: 95^0C-30 seconds and 65^0C for 10 min. This study was approved by the Department of Biotechnology, Government of India – authorized Wobble Base Bioresearch Institutional BioSafety Committee (WBB-IBSC), which oversaw the biosafety aspects associated with this study.

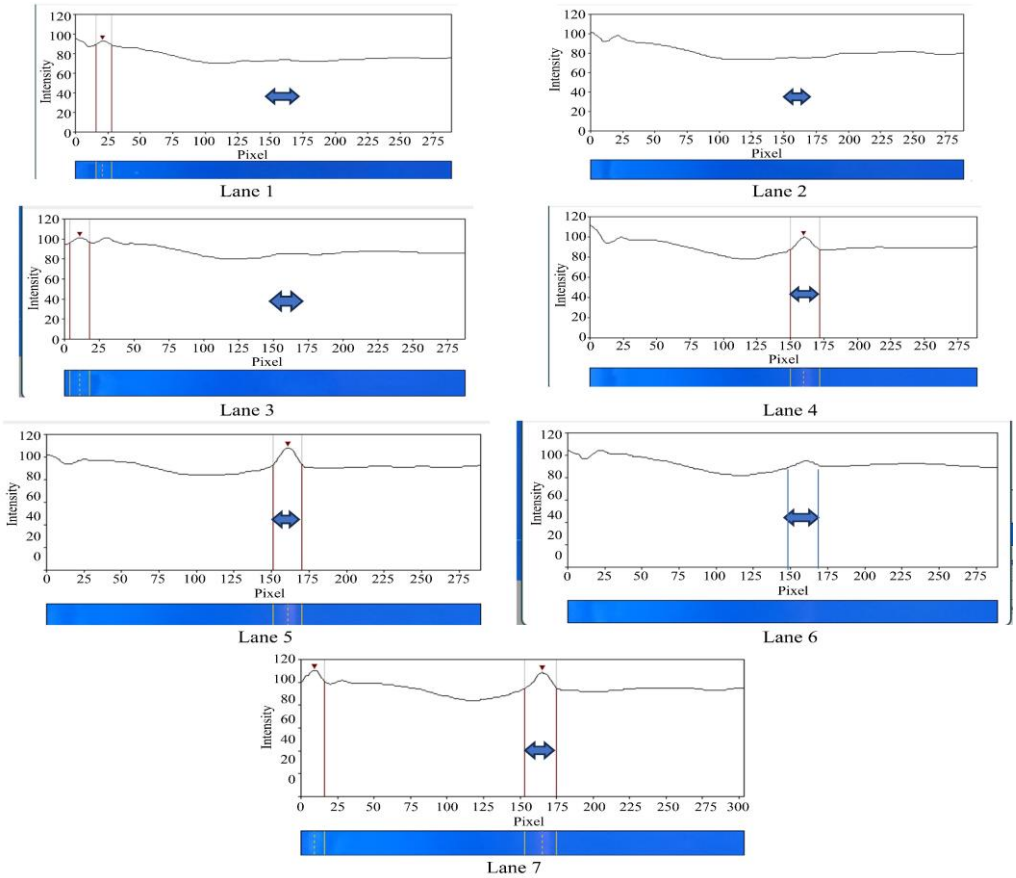


Fig. 1 Agarose gel lane scanning data using GelAnalyzer 23.1.1 software. Target bands were expected in the region between 150- and 175-Pixel points (X-axis) that corresponded to 221 bp and are shown in the graph using a double-headed arrow. No peaks were detected in Lane 1-3 of the agarose gel (data not shown) that corresponded to samples XLw-1 - XLw-3, while small peaks were detected in Lane 4-7 that corresponded to samples XLw-4 - XLw-7 respectively

3. Results

A set of 39 clinical samples, obtained from patients suffering from tuberculosis and having initiated 1st line therapy, were screened by Xpert MTB RIF Ultra assay to obtain a panel of 12 *M. tuberculosis*-positive samples. Of these, 7 (samples XLw-1–XLw-7) belonged to the 'MTB detected, low' category that translated to a low level of *M. tuberculosis* DNA available in the reaction.

DNA from sputum samples archived in the reagent-coated cellulose matrix and corresponding to these seven low-positive Xpert MTB Rif Ultra reports were extracted and subjected to end-point PCR (25 µL reaction volume) using the Internal Transcribed Spacer (ITS) region of the *M. tuberculosis* genome as the target. Out of the seven samples, four generated faint bands (Samples XLw-4–XLw-7) of the desired molecular weight, while the remaining three, labelled as XLw-1–XLw-3, did not generate any bands (Figure 1). Inhibition-check PCR using external DNA and its corresponding oligonucleotide primers generated amplicons in the presence of the test DNA solution, indicating an inhibition-free PCR (data not shown).

The DNA from the three end-point PCR-negative samples was then processed for LPA according to the prescribed protocol. The results indicated that one of them was faintly LPA-positive (XLw-2), whereas the remaining two (XLw-1 and XLw-3) were LPA-negative (Figure 2).

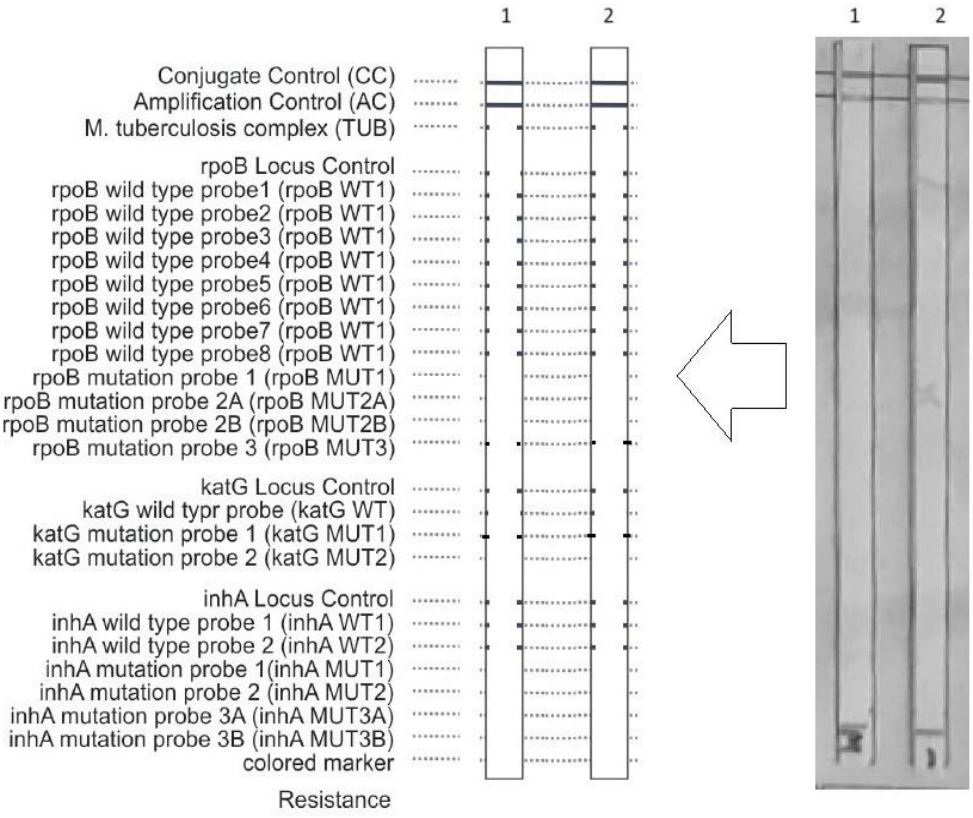


Fig. 2 Line Probe Assay (LPA) for sample numbers XLw-1 (Strip 1) and XLw-3 (Strip 2) generated an M. tuberculosis-negative result due to the low quantity of template DNA available for the assay. Only the conjugate and amplification controls are detected on top of the strips, while all other signals are absent. The description on the extreme left indicates the interpretation of each band that is visible when the optimal quantity of template DNA and the corresponding mutation is present or absent. The representative image in the middle is a sketch of the true result generated on the strip (extreme right)

Five microliters of the stock DNA from these two LPA-negative samples (XLw-1 and XLw-3) were subjected to whole genome amplification (WGA), and 5 µL of the whole genome amplified product was directly used for LPA. Five microliters of the WGA product were also subjected to end-point PCR (25 µL reaction volume) using ITS-specific primers, and 5 µL of the PCR-amplified product was resolved by agarose gel electrophoresis. Intense bands of the expected size (221 bp) were detected in both samples, and their band intensities were significantly high (Figure 3, Panel A and Panel B).

Scanning of the lanes of agarose gel corresponding to the samples showed nil Pixel units for samples XLw-1 to XLw-3, 108, 110, 104, and 110 for samples XLw-4 to XLw-7, and 160 and 174 for whole genome amplified XLw-1 and 3 samples, respectively (Figure 3, Panel C).

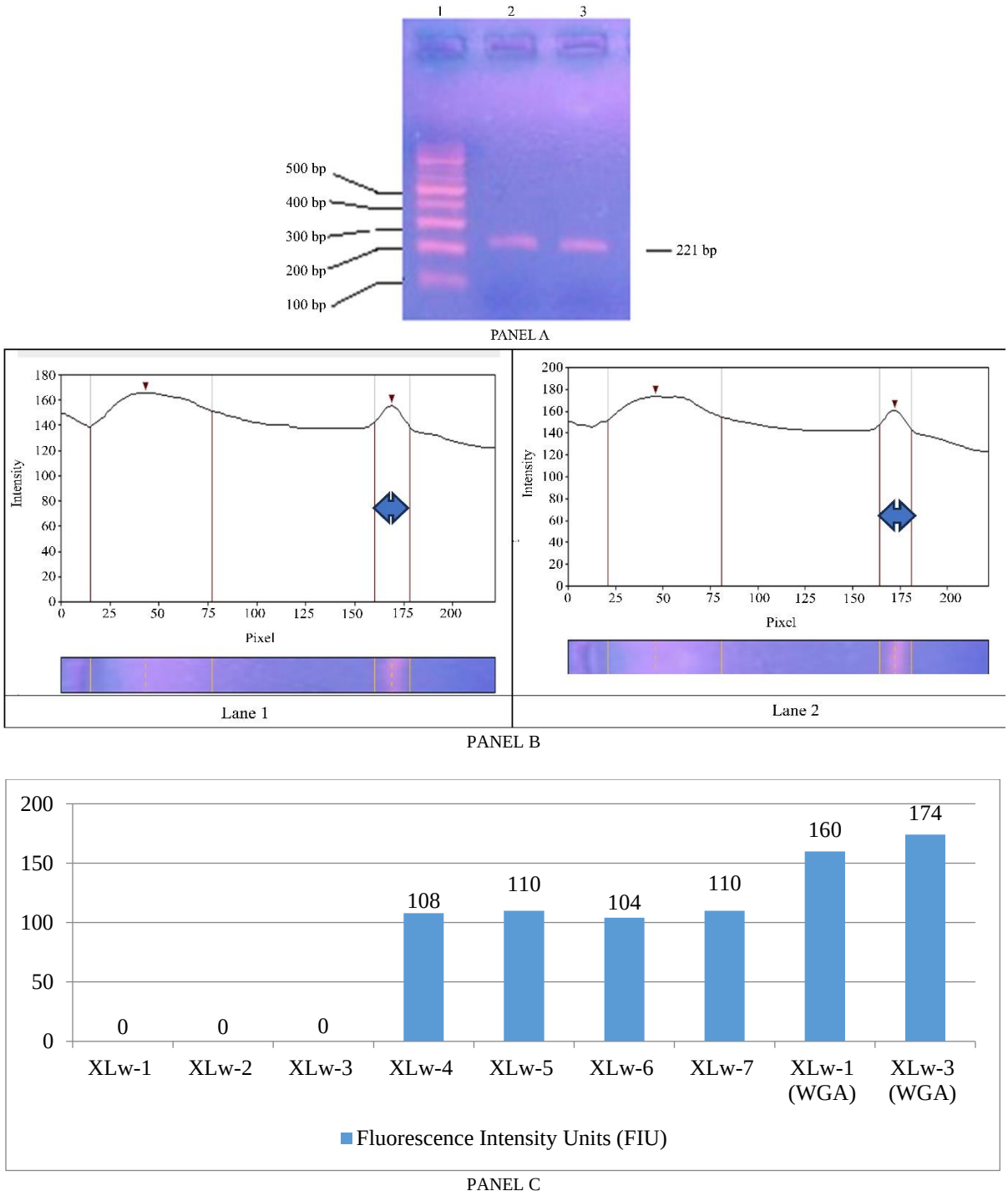
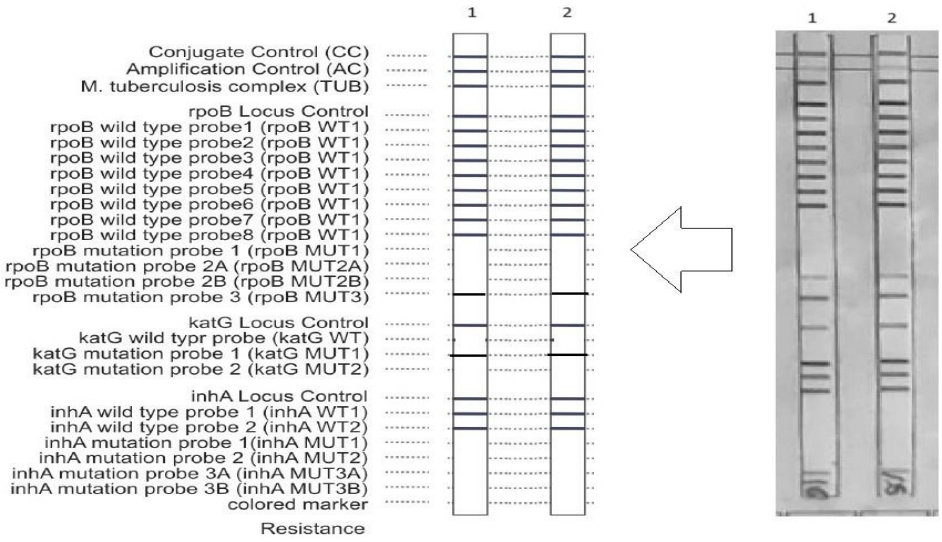
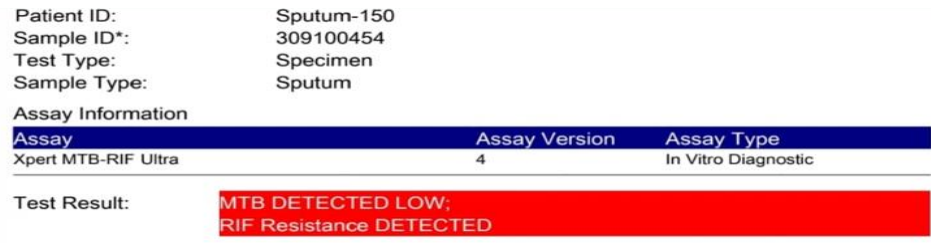


Fig. 3 Agarose gel electrophoresis analysis of 5 µL of Whole Genome Amplified products (total reaction volume: 25 µL) resolved on agarose gel electrophoresis for sample no XLw-1 and XLw-3 respectively. Panel A: Lane 1: 100 bp DNA size standard; Lane 2 & 3: Whole genome amplified products of samples XLw-1 and XLw-3, respectively. Panel B: Agarose gel (Panel A, this figure) lane scanning data using GelAnalyzer 23.1.1 software. Target bands were expected in the region between 162- and 177-Pixel points (X-axis) that corresponded to 221 bp and are shown in the graph using a double-headed arrow. Peaks were detected in Lane 1 and 2 that corresponded to the whole genome amplified product of samples XLw-1 and XLw-3, respectively. Panel C: Graphical representation of the Fluorescent Intensity Units obtained from scanning of agarose gel electrophoresis lanes when endpoint PCR amplification products targeting the ITS (Internal Transcribed Spacer) region of the M. tuberculosis genome were resolved. X-axis: Sample XLw-1 to XLw-7 - Fluorescence obtained from agarose gel electrophoresis (Figure 1, Panel A); Sample XLw-1 (WGA) and XLw-3 (WGA) -Fluorescence obtained from agarose gel electrophoresis when whole genome amplified product of sample XLw-1 and 3, labelled as XLw-1 (WGA) and XLw-3 (WGA) were resolved. In all cases, 5 µL of the products were resolved from a 25 µL PCR or whole genome amplified reaction volume.

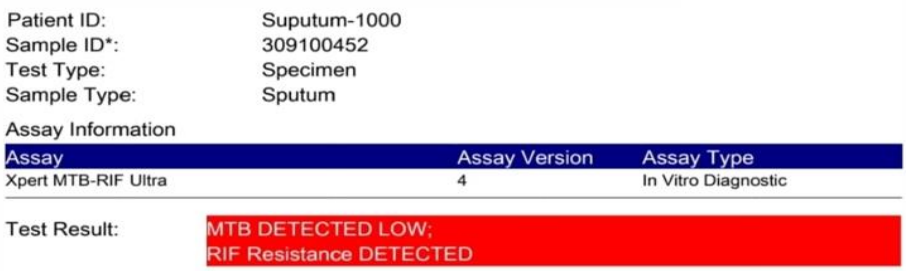
Samples XLw-1 and XLw-3 were also found to be LPA-positive after whole genome amplification, and their drug sensitivity profiles matched the original Xpert MTB RIF Ultra test profile of the samples (Figure 4).



PANEL A



Sample: ZLw-5



Sample XLw-7

PANEL B

Fig. 4 Results of Line Probe assay (LPA) of samples XLw-1 and XLw-3 after 5 µL of the original stock DNA released from cellulose matrix was subjected to Whole genome Amplification in a total reaction volume of 25 µL and 5 µL was used for running LPA. Panel A: Line Probe Assay (LPA) for Whole Genome Amplified version of sample number XLw-1 (Strip 1) and XLw-3 (Strip 2), which generated an *M. tuberculosis*-positive result with a drug-resistant mutation corresponding to rpoB mutation probe 2B. The description on the extreme left indicates the interpretation of each band that is visible. The representative image in the middle is a sketch of the true result generated on the strip (extreme right). Panel B: Clip showing Xpert MTB Rif Ultra report of the original stock of samples XLw-1 and XLw-3, respectively. The mutation profile observed in Panel A and Panel B for the samples XLw-1 and XLw-3 are identical, the difference being that in Panel A, the data was obtained after Whole Genome Amplification of the stock DNA, while in Panel B, the data was obtained from the original stock DNA.

The LPA reaction generated positive data when 5 µL of Dilution-1 samples (*M. tuberculosis* DNA of 125 ng spiked into *M. tuberculosis-negative* sputum DNA) were processed. However, when the same volume of dilution-2 DNAs (*M. tuberculosis* DNA of amount 1.25 ng spiked to *M. tuberculosis-negative* sputum DNA) was processed, LPA returned negative sample data. When 5 µL of dilution-2 DNA was subjected to whole genome amplification, and the same volume (5 µL) of the amplified products was used to run LPA. The result was positive ((Figure 5), and the resistance/sensitivity profile of the samples was identical to that obtained from the Dilution-1 sample (Figure 5).

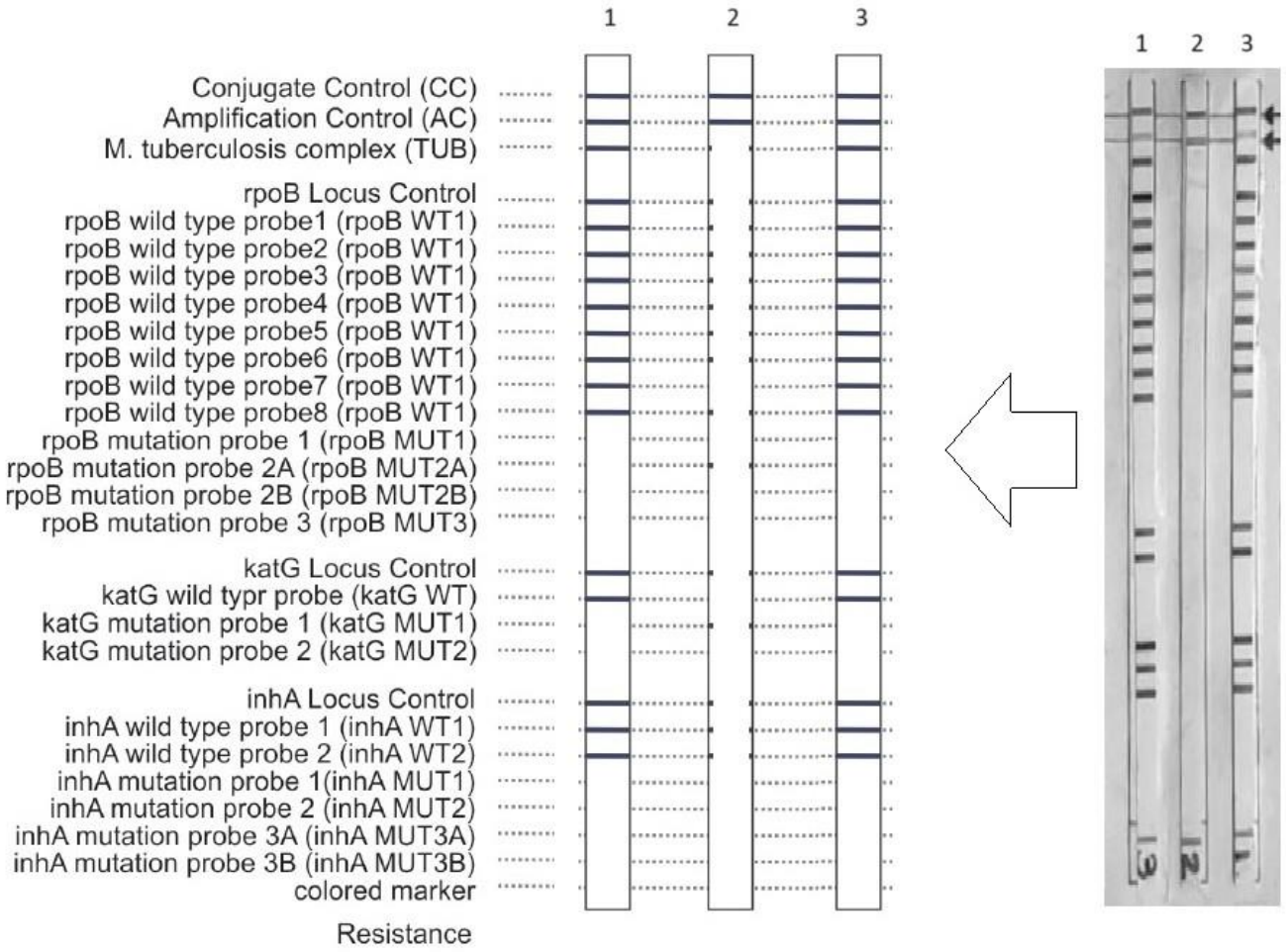


Fig. 5 Results of Line Probe Assay (LPA) for Dilution 1 (Strip 1), Dilution 2 (Strip 2) and Whole Genome Amplified Dilution 1 samples. Dilution 1 sample (125 ng M. tuberculosis DNA; Strip 1) and Whole genome Amplified Dilution 2 sample (Strip 3) generated identical M. tuberculosis-positive results, while Dilution 2 (1.25 ng M. tuberculosis DNA; Strip 2) failed to generate M. tuberculosis-positive results. The description on the extreme left indicates the interpretation of each band that is visible when the optimal quantity of template DNA and the corresponding mutation is present or absent. The representative image in the middle is a sketch of the true result generated on the strip (extreme right). The dilution samples were formulated by adding a precise quantity of DNA extracted from a laboratory culture of the M. tuberculosis strain to DNA extracted from the sputum of healthy individuals

4. Discussion

Simultaneous testing of isoniazid and rifampicin resistance when the diagnosis of tuberculosis is performed, is perhaps the most economical approach in all settings to increase the rate of cure, reduce mortality, and prevent the emergence of drug resistance in cases of tuberculosis by reducing the chances of failure and relapse [14]. In the past few decades, tuberculosis diagnosis has seen a mammoth revolution in molecular diagnostic techniques to detect sensitive and resistant variants of *Mycobacterium tuberculosis*. The threatening emergence of drug resistance in tuberculosis poses a strong challenge to the effective delivery of healthcare solutions at a global level and calls for not only the development of new antimicrobials but also the detection of drug-resistant variants of the pathogen in a way that is easily accessible, economical, and sensitive [15].

The Line Probe Assay, or LPA, is a promising solution for the rapid detection of *M. tuberculosis* drug resistance. It exploits the principles of multiplex polymerase chain reaction and reverse hybridization assays [16]. The World Health Organization (WHO) has endorsed the adoption of LPA in

screening and diagnostic algorithms for pulmonary tuberculosis in countries with a high burden of the disease [16].

The collection of clinical samples for tuberculosis diagnosis continues to remain an under-focused aspect in the entire ecosystem of molecular diagnosis of the disease.

Numerous challenges are associated with conventional methods of collecting, transporting, and storing sputum samples before molecular diagnosis [17]. These range from the leakage of infectious samples to extensive contamination during long transit. In this scenario, a solid matrix for room-temperature transportation and storage, coupled with the easy release of DNA present within the heterogeneous cellular population of the clinical sample trapped onto it, is a useful and viable alternative [9].

It is, therefore, important to demonstrate the seamless integration of such solid-support DNA storage devices with detection technologies such as LPA so that tuberculosis diagnosis gains further strength and can cover larger geographical areas of countries with a high disease burden, such as India. This study uses such a sputum transportation, storage, and DNA release device [9], [10] and demonstrates its integration with LPA *via* a short but effective whole-genome amplification step that significantly enhances the sensitivity of LPA.

Hain Lifescience (GmbH, Nehren, Germany), manufacturer of a popular LPA product for detecting drug-resistant tuberculosis, recommends a volume of 5 µL of DNA with a concentration ranging between 20-50 ng/µL as the starting material for running the assay. Therefore, a total amount of 100-250 ng of DNA is recommended for the proper functioning of the assay. However, obtaining an M. tuberculosis-positive sample at the optimal LPA concentration is not always feasible, which can increase the probability of generating false negatives on this platform.

In order to address this issue, an approach was adopted wherein a sub-optimal quantity of DNA (with regard to LPA assay) released from a solid support, as described by Goyani et al. (2023) [4],[5], was amplified using *Bst* DNA polymerase, wherein its strong strand displacement activity was exploited to achieve long and continuous stretch of amplified DNA in a short period of time.

Other popular methods of whole genome amplification, such as Multiple Displacement Amplification (MDA) using phi29 DNA polymerase and Degenerate Oligonucleotide-Primed PCR (DOP-PCR) employing degenerate primers that prime randomly across the genome [18], are available and are used more frequently in the molecular biotechnology field, the current method proved to be effective in this particular case owing to the small size of the target genome compared to that in human cells, which were preferentially amplified during the process.

The experiment required clinical sputum samples with low bacterial pathogen loads. Earlier studies indicated that the bacterial load of *M. tuberculosis* in sputum samples of patients under treatment is low, corresponding with improved clinical conditions and reduced transmission risk [19]. This was the rationale behind the specific composition of the resource population used in this study, where all samples were obtained from tuberculosis patients undergoing 1st line of therapy (Isoniazid, Rifampicin, Pyrazinamide, and Ethambutol or HRZE) [20].

Screening of 39 patients yielded seven samples that recorded low" when tested on the Xpert MTB RIF Ultra assay. This invariably indicated a low load of pathogens in the clinical sample and, hence, low pathogen DNA in the corresponding extracted DNA samples. This observation was reflected when the extracted DNA from these seven samples (XLw-1 to XLw-7) were subjected to traditional end-point PCR targeting the ITS region of the pathogen genome. As expected, three samples (XLw-1 to XLw-3) failed to generate any detectable PCR amplicons when resolved on an agarose gel, whereas the remaining four samples (XLw-4 to XLw-7) generated faint bands (Figure 1). This result was expected because previous studies have shown that *M. tuberculosis* DNA in sputum samples that generate low signals in the Xpert MTB RIF Ultra assay (or its variants) are most often not detectable by end-point PCR methods.

These three samples, *viz.*, XLw-1, 2 and 3, respectively, were used for further study and subjected to a Line Probe Assay. Our data showed that while the LPA test was weakly positive for sample XLw-2, samples XLw-1 and XLw-3 were reported as false negatives (Figure 2). This observation aligns with earlier findings that indicated that the sensitivity of line probe assays for detecting *M. tuberculosis* genome targets is less than that of fluorescent real-time PCR methods [21].

When the stock DNA of samples XLw-1 and XLw-3 was subjected to whole-genome amplification followed by end-point PCR targeting the ITS region of the *M. tuberculosis* genome, intense bands were detected (Figure 3). It may be noted that in all agarose gel electrophoresis experiments, a total volume of 5 µL of DNA was loaded after adding 2 µL of agarose gel loading dye (6X). Given that 5 µL of stock DNAs from samples XLw-1 and XLw-3 was used for whole genome amplification in a total volume of 25 µL and 5 µL of the whole genome amplified product was resolved in the agarose gel, it effectively corresponded to 1/5th or 1 µL of the stock DNA. The relative fluorescence of the PCR amplicons after agarose gel electrophoresis indicated that the intensity of the whole genome amplified products (post-WGA, DNA-amplified products; sample XLw-1 WGA and XLw-3 WGA) was 160- and 174-Pixel units compared to XLw-4, 5, 6, and 7 samples

(pre-WGA stage, stock DNA-amplified products), where the band intensity ranged between 104 and 110-pixel units and nil in the case of samples XLw-1, 2, and 3 (pre-WGA stage, stock DNA-amplified products) (Panel C, Figure 3).

This showed that the genomic region targeted by conventional end-point PCR was sufficiently amplified during the whole genome amplification process and provided the desired quantity of template DNA for successful PCR amplification in samples XLw-1 (WGA) and XLw-3 (WGA).

Whole genome amplification (WGA) employing Bst DNA polymerase occurs through Multiple Displacement Amplification (MDA), which is known to be a highly efficient process for amplifying small quantities of DNA into microgram levels with a high degree of fidelity. This method has been previously demonstrated for whole-genome amplification in other biological samples [22].

After agarose gel electrophoresis, the whole genome amplified products corresponding to samples XLw-1 and XLw-3 were subjected to LPA. The results indicated that both amplified samples effectively generated positive LPA results and that the drug resistance profile matched that obtained from Xpert MTB Rif Ultra tests performed on the original stock DNA of these two samples (Figure 4).

To confirm the effectiveness of the whole genome amplification step, DNA extracted from the sputum of healthy individuals was spiked with *M. tuberculosis* DNA extracted from *M. tuberculosis* bacilli cultured in the laboratory in LJ medium [23]. DNA extracted from human sputum samples contains both mycobacterial and human DNA, which can cause complications in molecular diagnostic processes during downstream analysis [24]. Therefore, to mimic a typical infected sputum sample, *M tuberculosis* DNA-spiked healthy sputum DNA was prepared.

The results indicated that a positive LPA result was obtained in sample coded Dilution-1, where the total M. tuberculosis DNA available for the LPA reaction was 125 ng. However, a false-negative report was generated when the sample coded Dilution-2 containing 1.25 ng of total M. tuberculosis DNA was subjected to LPA.

When 5 µL of Dilution-2 DNA was subjected to 10 min of whole genome amplification, and 5 µL of the amplified product was used to run an LPA, the result was positive, and the drug resistance/sensitivity profile matched that of the Dilution-1 sample. This indicates that the amplification of specific targets within the whole genome of *M. tuberculosis* present in the Dilution-2 formulation occurred effectively and efficiently to return a positive LPA result.

Based on the findings presented in this paper, it is evident that addressing the challenge of low sensitivity in the Line Probe Assay (LPA) compared to fluorescent real-time PCR methods can be effectively achieved through the innovative use of sputum DNA archived in chemically coated solid cellulose matrices. The matrix facilitates room temperature transportation and extended storage of clinical samples, while its ultra-short DNA release step and brief whole-genome amplification protocol efficiently amplify low quantities of *M. tuberculosis* DNA present in these samples. The application of this technology not only enhances the sensitivity of *M. tuberculosis* LPA but also has the potential to convert false negative results into true positive outcomes. This approach represents a significant advancement in diagnostic capabilities, particularly in resource-limited settings where maintaining cold chain logistics is challenging. By improving LPA's detection limit and reliability, sputum transportation, storage, and DNA-release technology integration promise to diagnose and manage tuberculosis positively.

5. Conclusion

In conclusion, leveraging the cellulose matrix technology for room temperature transportation, extended storage, and enhanced DNA release and its integration with a brief whole-genome amplification protocol offers a promising solution to enhance the sensitivity of *M. tuberculosis* LPA, thereby improving the accuracy and effectiveness of tuberculosis diagnostics.

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