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ORIGINAL RESEARCH ARTICLE

Establishment and evaluation of a naked-eye diagnostic assay for tuberculosis utilizing reverse isothermal amplification-assisted CRISPR-Cas in resource-limited settings

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ABSTRACT

Introduction: The current scenario of tuberculosis (TB) caused by *Mycobacterium tuberculosis* (MTB) has presented an almost insurmountable challenge to hospitals with high patient numbers. Delayed diagnosis of TB is a major hurdle in preventing the employment of efficient therapeutics, leading to the development of drug resistance. Hence, an easily accessible diagnostic method, particularly for resource for resource-limited settings, is pertinent for the rapid identification of MTB-infected patients. In pursuit of developing such an assay, the present study offers a CLAP-TB (CRISPR-Cas coupled RT-LAMP Amplification Protocol for Tuberculosis) assay, which will allow us to diagnose TB rapidly and visually.

Methods and results: Herein, the visual MTB detection consists of a method utilizing 232 different samples (sputum, urine, serum) from 82 patients for reverse transcription loop-mediated isothermal amplification (RT-LAMP). Additionally, the assay also utilizes the integration of a CRISPR-Cas12-based system using different guide RNAs of *IS6110* and an internal control *POP7* (human RNase P) genes along with visual detection via lateral flow readout-based dipsticks with the unaided eye (~134 min). Overall, the limit of detection for CLAP-TB assay was up to 1 ag of RNA, while the clinical sensitivity and specificity were 98.27% and 100%, respectively, on the pilot scale.

Conclusion: Together, our CLAP-TB assay offers proof of concept for a rapid, sensitive, and specific method with the minimum technical expertise required for TB diagnosis in developing and resource-limited settings.

Keywords: CRISPR-Cas12, Lateral flow readout, *Mycobacterium tuberculosis*, RT-LAMP, Tuberculosis

Introduction

Mycobacterium tuberculosis (MTB), which is the causative agent for tuberculosis (TB), affects around one-third of the world population and continues to wreak havoc across the globe, leading to unimaginable loss of human lives (1,2). The healthcare infrastructure of several countries, particularly Asian countries, is overburdened. This evocative scenario of TB is leading to deaths because of a lack of quick diagnosis and effective treatment. Some drugs viz. Rifampicin and Isoniazid are available in the regimen, but it takes around 4-6 months for the proper treatment; thus, the emergence of drug resistance becomes another obstacle against efficient therapeutics (3,4). Although the lung is the most common site of infection, mycobacteria can infect extrapulmonary sites, leading to extrapulmonary tuberculosis (EPTB) accounting for almost one-fourth of TB cases.

Additionally, diagnosis of EPTB is more challenging as specimens of EPTB (such as cerebrospinal and pleural fluids) are not readily available by non-invasive methods used to obtain sputum and are less sensitive (5,6). Among current diagnostic assays, culture-based diagnostic methods are slow, time-consuming, and less sensitive, while PCR-based methods are relatively faster but costly (7-9). Therefore, the development of simple yet sensitive diagnostic methods is needed for the diagnosis of efficient epidemiological management of TB patients.

Loop-mediated isothermal amplification (LAMP) is a nucleic acid-based detection method that is efficiently used for human pathogens (viruses, bacteria, and malaria), including TB, but suffers the drawback of false positive and specificity issues. Additionally, LAMP-based methods fail to detect viable MTB cells even though numerous LAMP-based methods are available in clinical diagnostics labs (10). CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)/Cas system is a genome editing tool that is utilized in molecular diagnostic systems. CRISPR, with precision and

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specificity at a single nucleotide level, can identify the target nucleic acid with the help of a guided nucleotide sequence (gRNA) (11,12). Cas enzyme leads to indiscriminate cleavage activity, and this property is utilized to develop nucleic acid endonuclease-targeted CRISPR trans reporter (DETECTR) system for targeting nucleic acid detection.

In the current study, we have developed a CLAP-TB (CRISPR-Cas coupled RT-LAMP Amplification Protocol for Tuberculosis) assay, which will allow rapid (~134 min) and visual diagnosis of TB. The CLAP-TB assay involves a method for the detection of MTB from various clinical samples such as sputum, urine, and serum that can visually be detected with an unaided eye. CLAP-TB is based on the integration of RT-LAMP with the CRISPR-Cas technique (to take care of false positive and specificity issues) that allows the faster detection of *Mycobacterium* cells from patient samples with high accuracy within one hundred and thirty-four minutes. Taken together, we have developed a simple, rapid, sensitive, and specific assay to visually diagnose MTB from sputum as well as other body fluids. This would facilitate the onsite usage for enhanced testing throughout the country, particularly in places with limited resources, such as those present in tier II and tier III locations. Given the challenges associated with TB detection, such as the lack of successful therapeutics and delayed diagnosis, this assay would greatly facilitate visual TB detection based on lateral flow strips and help save valuable human lives.

Materials and methods

Clinical sample collection and decontamination

The patient samples and Healthy control samples were collected at All India Institute of Medical Sciences (AIIMS), Bhopal. The samples were collected in two categories and processed accordingly. (i) Specimens like sputum and urine were collected under sterile conditions to avoid contamination. (ii) In other patient groups, blood samples were collected for serum required for the method development, evaluation, and validation. A total of 232 clinical samples were collected based on their Ziehl-Neelsen (ZN) staining, Acid-fast bacilli (AFB) culture, and GeneXpert reports, where 161 were true positive patient samples (PS), and 71 were true negatives considered as negative controls (NS), which also included 20 healthy controls (serum and urine).

The clinical specimens received from the patients were first decontaminated by the NALC-NaOH method (modified Petroff's method) (13), and the smear was prepared for the ZN staining from the direct and decontaminated specimens. In brief, about 5-6 mL contaminated specimen was mixed with an equal volume of 0.5% NALC – 4% NaOH mixture in a 50 mL Oakridge capped round bottom processing tube; the mixture was vortexed and incubated at 37°C for 10 minutes. After incubation, the mixture was neutralized with phosphate buffer (pH 6.8) up to 50 mL total volume, followed by centrifugation at 8000× g for 10 minutes. The pellet was resuspended in 2 ml phosphate buffer (pH 6.8). From the decontaminated sample, 0.2 mL of suspension was used to isolate RNA. The samples were properly labeled and placed in a cryo box and further kept in a leak-proof box containing dry ice for subsequent transfer to the Biosafety laboratory of Amity University Haryana.

RNA isolation

RNA was isolated from MTB using the RNeasy Mini Kit (Qiagen, Germany) (14) in accordance with the manufacturer's instructions and treated with DNase I. Separately, purified genomic DNA was also used as a negative control for validation of the proposed assay to ensure any non-specific amplification. The isolated RNA was visualized for size and integrity by agarose gel electrophoresis. The concentration of purified RNA was measured spectrophotometrically using NanoDrop™ (ThermoFisher) and diluted in nuclease-free water to working concentrations.

Primers, lateral flow reporters, and guide RNAs

Two different primers set for RT-LAMP were used in this study, one for the *IS6110* gene transcript and one for the *POP7* gene transcript (Table 1). The primers for all the genes were designed using the NEB primer designing tool. These regions were further modified to meet the requirements for RT-LAMP. Similarly, compatible gRNAs were designed to target the *IS6110* and *POP7* genes. The lateral flow reporter (/56-FAM/TTATTATT/3Bio/, IDT) used in this study was previously reported (14,15).

RT-LAMP Reaction

The corresponding LAMP primer group consists of the six specific primers with their stock concentrations as: an upstream outer primer F3 (2 µM), a downstream outer primer B3 (2 µM), an upstream inner primer FIP (16 µM), a downstream inner primer BIP (16 µM), an upstream ring primer LF (8 µM) and a downstream ring primer LB (8 µM). The 10× primer mix of 50 µL was prepared by using the above-mentioned primers and then used for the RT-LAMP reaction. The RT-LAMP reaction was performed by using the New England BioLabs Protocol (Online). A total volume of 12.5 µL was used to set the RT-LAMP reaction. The reaction was prepared by using 6.25 µL of 2× Warmstart Colorimetric Master mixes as per the manufacturer guidelines comprising Bst Polymerase and reverse transcriptase enzyme in it, 1.25 µL of 10X Primer mix containing RT-LAMP primers and one µl of purified RNA with 2.5 µL of NFW. Reactions were performed separately for *ISS6110* and *POP7* genes at 65°C for 40 minutes in a water bath. In the NEB Warmstart master mix, one component is phenol red dye (indicator for pH change), which changes from pink to yellow as the pH changes. During the amplification, protons (H^+) ions are released as a byproduct of dNTPs hydrolysis. This lowers the pH and causes the color to change from pink to yellow.

CRISPR-Cas12 assay

Nucleoprotein complex

The nucleoprotein complex (Cas12a-gRNA complex) is formed as Lb Cas12a, a site-specific DNA endonuclease guided by a single 41-44 nucleotide guide RNA (gRNA). This targeting requires a gRNA complementary to the target site

TABLE 1 - List of primers and guide RNA sequences used in the study

Primers/gRNAs	Sequences and modifications	Length	Genes
F3	5′-CCGCGGTCAGCACGATT-3′	17 nt	IS6110
B3	5′-CGACGCGGTCTTTAAAATCG-3′	20 nt	IS6110
FIP	5′-TTACGCACCGTCTCCGCGCTTTTAGTGGGCAGCGATCAGT-3′	40 mer	IS6110
BIP	5′-CCGGGACCACGACCGAAGATCGCAATTCGGCGTTGTC-3′	37 mer	IS6110
LF	5′-CTGAGCTGAAGCGCTTGC-3′	19 nt	IS6110
BF	5′-CTGAGCTGAAGCGCTTGC-3′	18 nt	IS6110
gRNA	5′-UAAUUUCUACUAAGUGUAGAUAGUGGGCAGCGAUCAGUGAG-3′	41 mer	IS6110
F3	5′-TTGATGAGCTGGAGCCA-3′	17 nt	RNase POP 7
B3	5′-CACCCTCAATGCAGAGTC-3′	18 nt	RNase POP 7
FIP	5′-GTGTGACCCTGAAGACTCGGTTTTAGCCACTGACTCGGATC-3′	41 mer	RNase POP 7
BIP	5′-CCTCCGTGATATGGCTCTTCGTTTTTTTCTTACATGGCTCTGGTC-3′	45 mer	RNase POP 7
LF	5′-ATGTGGATGGCTGAGTTGTT-3′	20 nt	RNase POP 7
BF	5′-CATGCTGAGTACTGGACCTC-3′	20 nt	RNase POP 7
gRNA	5′-UAAUUUCUACUAAGUGUAGAUAAUUACUUGGGUGUGACCCU-3′	41 mer	RNase POP 7

as well as a 5′ TTTN or TTN protospacer adjacent motif (PAM) on the DNA strand opposite the target sequence. The cleavage by LbCas12a occurs ~18 bases 3′ of the PAM and leaves 5′ overhanging ends. For the formation of nucleoprotein complex, a final volume of 25 µl was prepared by using 16 µl of LbCas12a enzyme. In 1X NEB buffer 2.1, 3µl gRNA of *IS6110* and *POP7* genes, 2.5 µl of 10X NEB buffer 2.1, and 3.5 µl of nuclease-free water (NFW) kept at 37°C for 30 minutes inside an incubator. The complex was used immediately or stored at 4°C for further use.

Trans-cleavage reaction

After the formation of the nucleoprotein complex (Cas12a-gRNA complex), 100µl of reaction was set up with 10µl of 10X NEB buffer 2.1, 2 µl of RT-LAMP product, 50nM of lateral flow reporter, 18 µl of nucleoprotein complex, and 65 µl of NFW. The reaction was incubated at 37°C for 60 minutes in an incubator.

Lateral flow readout

To check the trans cleavage reaction, we use the lateral strips from Milenia Hybrid (Cat no MGHD 1), which are coated with gold nanoparticles and anti-FAM antibodies. Around 15 µL of the trans-cleavage reaction mixture was applied on the sample application area of the lateral flow strip, and the sample application area of the strip was placed into 60 µL of buffer in an upright position. The result was visualized after approximately 3 min. A single band (control band) close to the sample application area indicated a negative result, whereas a single band (test band) close to the top of the strip or the appearance of both bands indicated a positive result. Reactions were performed separately for *IS6110* and *POP7* genes.

The lateral-flow readout chemistry is based on the cleavage of a FAM-biotin reporter by Cas12a enzyme (14,16). The reporter is labeled with FAM at one end and biotin at another end. The streptavidin, which specifically binds to biotin, is present on the C-line (control), and the anti-rabbit antibody (FAM antibody) is present on the T-line (Test). When there is no collateral cleavage, the dual-labeled reporter remains intact, and the biotin is bound by the streptavidin, while the FAM label is bound by the mobile anti-FAM antibodies conjugated to the gold nanoparticles. This results in a strong C-line and the absence of a T-line. When there is collateral cleavage, the biotin-free FAM-labelled probe bound by the anti-FAM antibody moves and develops on the T-line, giving a positive test band.

Statistical analysis

The statistical analysis for the CLAP-TB assay was calculated according to the following equations: TPR (True positive rate) = True Positive/(True Positive + False Negative). TNR (true negative rate) = True Negative/(False Positive + True Negative). FNR (False negative rate = False Negative/(True Positive + False Negative). FPR (False Positive Rate = False Positive/(False Positive + True Negative). The sensitivity and specificity of the CLAP-TB assay were calculated as described elsewhere (14,17).

Results

CLAP-TB assay for IS6110 gene

First, we sought to perform the RT-LAMP of MTB RNA for the gene *IS6110*. The amplification of MTB target *IS6110* gene RNA was visible from the naked eye by a color change from pink to yellow, contrary to the negative template control (NTC), which retained a pink color (Fig. 1a). This amplification was also validated by gel electrophoresis which showed a distinct banding pattern (Fig. 1b). Further, CRISPR-Cas reaction was deployed, and results were interpreted on lateral flow

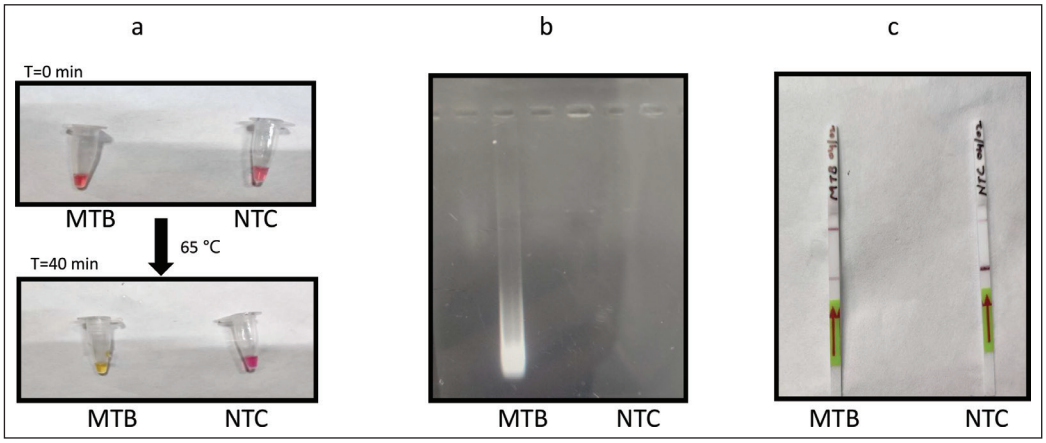

FIGURE 1 - CLAP-TB assay with MTB RNA (a) RT-LAMP reaction of *IS6110* gene for MTB RNA and NTC visualized by color change from pink to yellow after 40 min at 65°C. (b) The agarose gel image of the amplified product was obtained from the RT-LAMP reaction. (c) The lateral flow readout results of the *IS6110* gene for MTB and NTC after 3 min. NTC, non-template control.

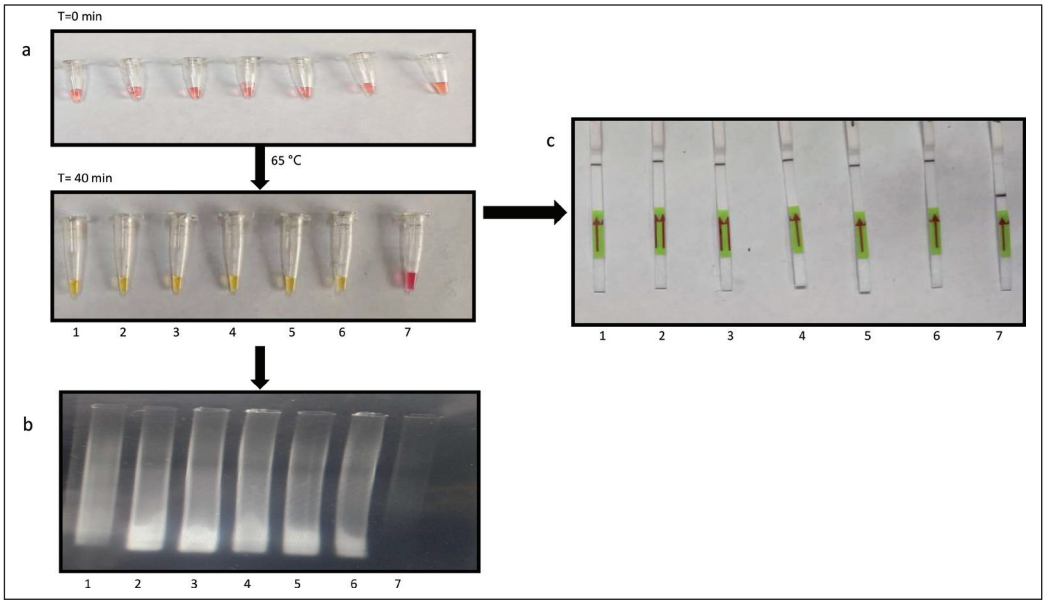

FIGURE 2 - Sensitivity of CLAP-TB assay (a) RT-LAMP reaction of *IS6110* gene for MTB RNA with various dilutions in lanes 1) 1000 fg, 2) 100 fg, 3) 10 fg, 4) 100 ag, 5) 10 ag, 6) 1 ag, and 7) NTC visualized by color change from pink to yellow after 40 min at 65°C. (b). The agarose gel image of the amplified product was obtained from the RT-LAMP reaction. (c) Lateral flow readout results for the sensitivity of CLAP-TB assay.

strips. We found that test band intensity was negligible in NTC, contrary to the MTB RNA depicting a clear test band (Fig. 1c).

Sensitivity of CLAP-TB assay

The limit of detection (LOD) for our CLAP-TB assay was determined using different dilutions of extracted RNA from pure cultures of MTB. LOD was considered the lowest dilution of RNA detected on the lateral flow strips by our CLAP-TB assay. We observed that on the lateral flow strip strips, the LOD for CLAP-TB was up to 1 ag of RNA (~1 copy) per reaction (Fig. 2). Next, we sought to compare the LOD of CLAP-TB with DNA as a template instead of RNA. We observed that LOD using DNA was only till 100 fg (Fig. 3a), contrary to 1 ag using RNA as a template (Fig. 3b).

Specificity of CLAP-TB assay

To evaluate the specificity of the CLAP-TB assay, we used four different strains viz. *Mycobacterium marinum*, *Mycobacterium smegmatis*, *Candida auris, Escherichia coli* other than MTB. The RT-LAMP reaction was performed at 65°C for 40 minutes using RNA as a template isolated from *M. marinum*, *M. smegmatis*, *C. auris,* and *E. coli.* The amplification of MTB target *IS6110* gene RNA was visible from the naked eye by a color change from pink to yellow contrary to the *M. marinum*, *M. smegmatis*, *C. auris, E. coli,* and negative template control (NTC), which retained pink color (Fig. 4a). This amplification was also validated by gel electrophoresis which showed a distinct banding pattern (Fig. 4b). Upon integration of CRISPR technology for the specific detection of MTB for the *IS6110* gene, we observed that in *M. marinum*, *M. smegmatis*, *C. auris, E. coli* and NTC, the test band intensity was negligible contrary to the MTB RNA *IS6110* gene depicting clear test band (Fig. 4c).

Validation of CLAP-TB assay with different clinical samples (Sputum, Urine, Serum)

After standardizing the CLAP-TB assay on MTB culture RNA, we sought to check the suitability of the developed assay from different clinical samples of TB patients. The collected sputum

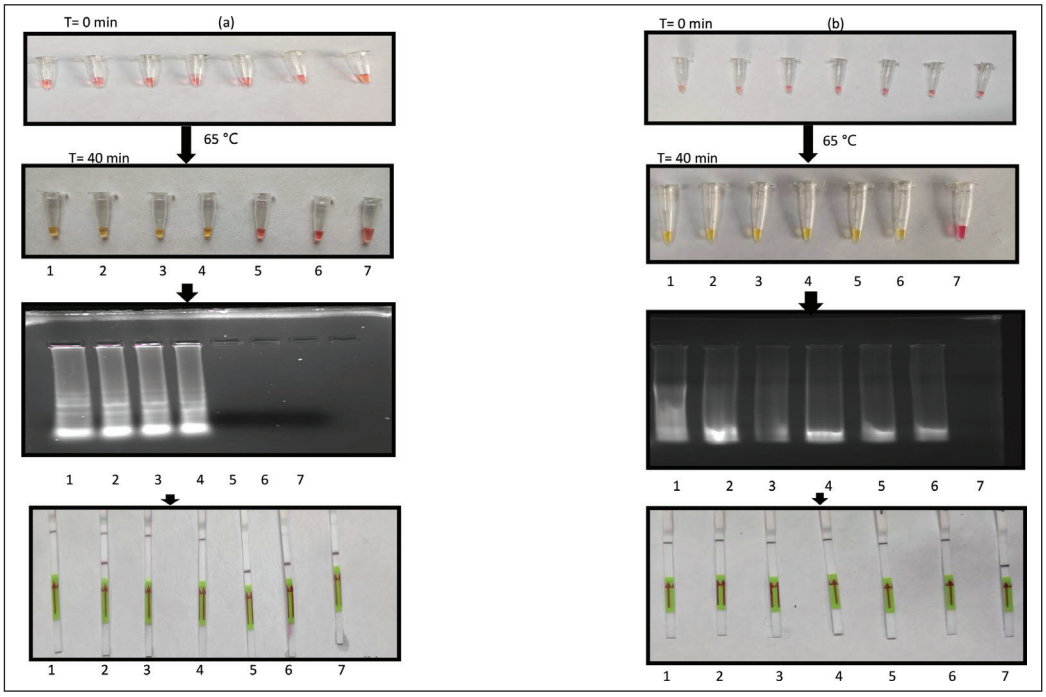

FIGURE 3 - Comparison of the sensitivity of CLAP-TB between DNA and RNA templates. LAMP and RT-LAMP reaction of *IS6110* gene for MTB with various dilutions of DNA (a) and RNA (b) in lanes 1) 1000 ng, 2) 1000 pg, 3) 1000 fg, 4) 100 fg, 5) 10 fg, 6) 1 ag, and 7) NTC visualized by color change from pink to yellow after 40 min at 65°C. The agarose gel image of the amplified product was obtained from LAMP (left panel) and RT-LAMP (right panel) reactions. Lateral flow readout results for sensitivity of CLAP-TB assay with DNA (left panel) and RNA (right panel).

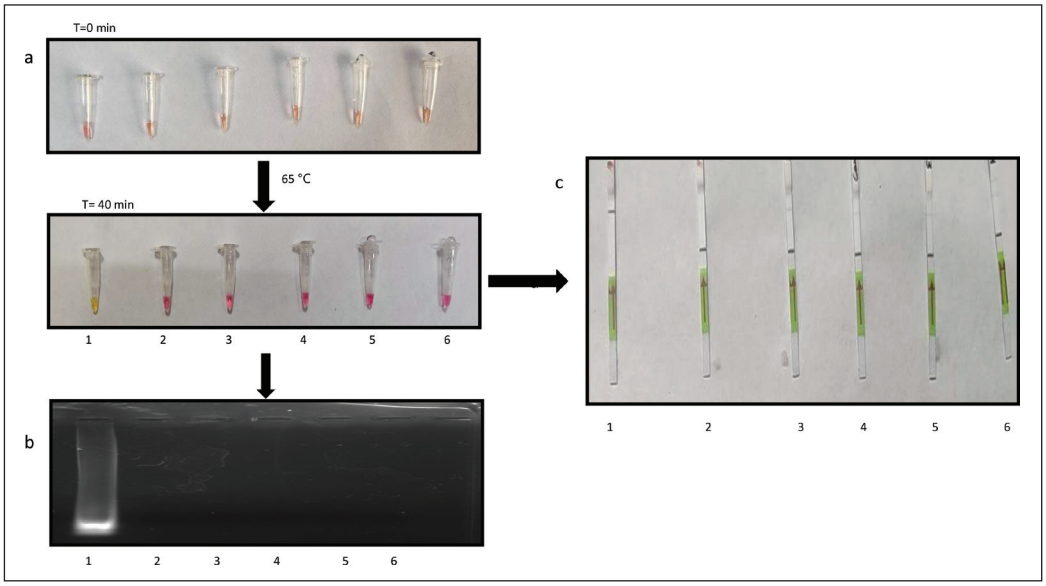

FIGURE 4 - Specificity of CLAP-TB assay (a) RT-LAMP reaction of *IS6110* gene from RNA of 1) MTB ($H_{37}R_v$), 2) *M. marinum,* 3) *M. smegmatis*, 4) *C. auris,* 5) *E. coli* and 6) NTC visualized by color change from pink to yellow after 40 min at 65°C. (b) The agarose gel image of the amplified product was obtained from the RT-LAMP reaction. (c) The lateral flow readout results of the *IS6110* gene for MTB, *M. marinum, M. smegmatis*, *C. auris, E. coli,* and NTC after 3 min.

(Fig. 5), urine (Fig. 6), and serum (Fig. 7) samples were utilized for the RT-LAMP reaction using *IS6110* gene primers. The color changes were observed in all positive clinical samples (PS), confirming positive amplification. There was no change in RNA isolated from patient samples (NS) that were negative for TB or NTC. Further, results were interpreted on lateral flow strips, and it was found that test band intensity was negligible in NTC, contrary to the MTB RNA isolated from sputum, urine, and serum samples, which depicted clear test bands.

POP7 gene (RNase P) used as an internal control for CLAP-TB assay

We utilized the *POP7* gene, which codes for RNase P and was used as an internal control for all positive and negative samples. *POP7* gene primers were used to set the RT-LAMP reaction on RNA isolated from PS, NS, and NTC. As expected, the color change was noticed for both PS and NS except the NTC, which was also confirmed by gel electrophoresis. Further, results were interpreted on lateral flow strips, and it was found that test band intensity was negligible in NTC, contrary to the MTB RNA isolated from positive and negative samples, which depicted clear test bands (Fig. 8).

Statistical validation of CLAP-TB assay

Lastly, we performed the statistical validation of the CLAP-TB assay as described in the methods. Out of the 232 different categories of clinical samples, 161 were true positive samples, and 71 were true negative samples, along with

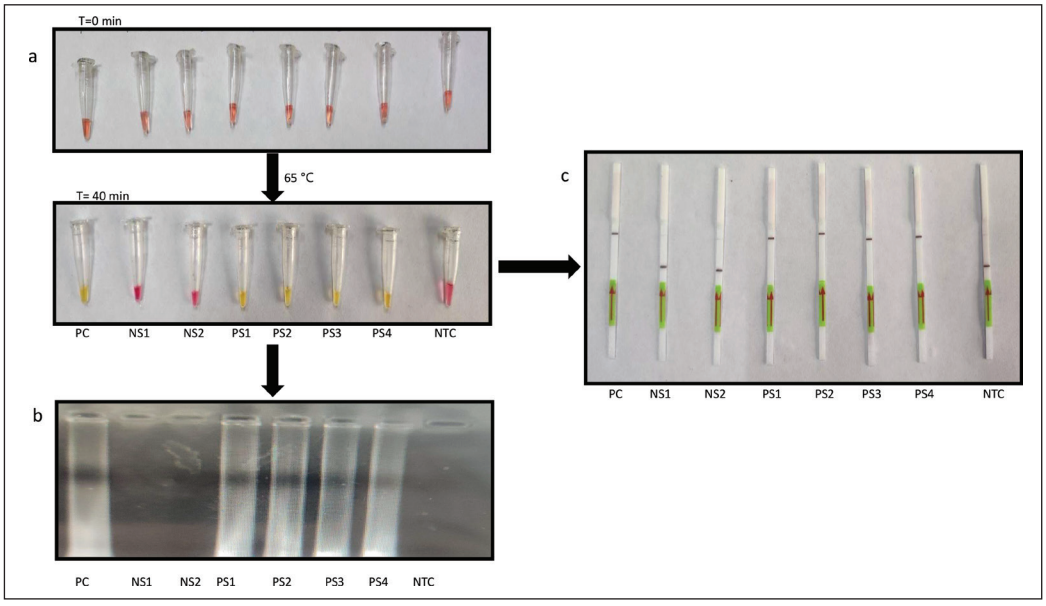

FIGURE 5 - CLAP-TB assay with clinical sputum samples (a) Representative RT-LAMP reaction of *IS6110* gene from MTB culture RNA as a positive control (PC), two negative samples (NS1 and NS2), four positive samples (PS1-4) and NTC visualized by color change from pink to yellow after 40 min at 65°C. (b) The agarose gel image of the amplified product was obtained from the RT-LAMP reaction. (c) The lateral flow readout results of the *IS6110* gene from MTB culture RNA as a positive control (PC), two negative samples (NS1 and NS2), four positive samples (PS1-4), and NTC.

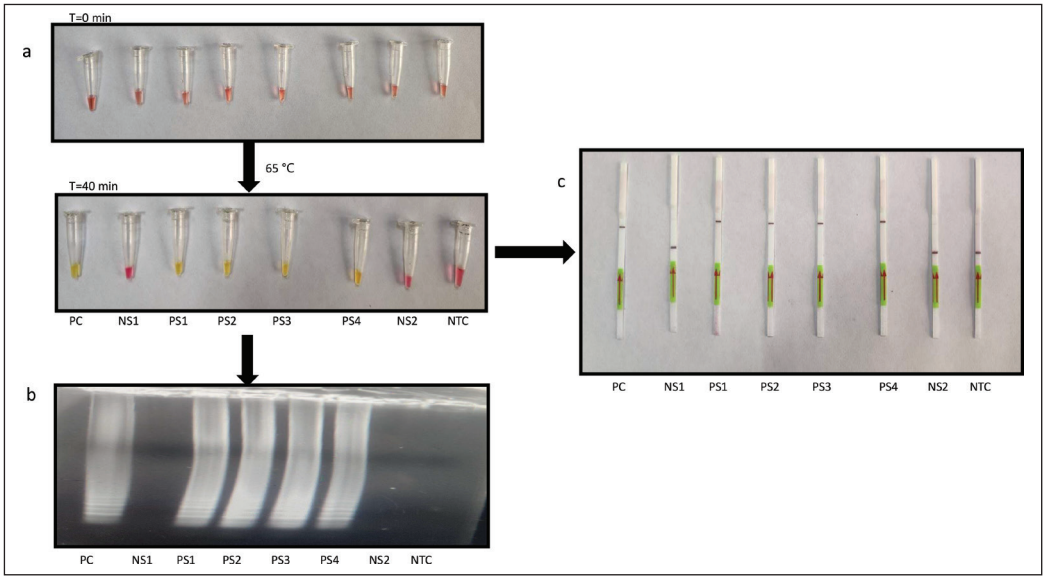

FIGURE 6 - CLAP-TB assay with clinical urine samples (a) Representative RT-LAMP reaction of *IS6110* gene from MTB culture RNA as a positive control (PC), two negative samples (NS1 and NS2), four positive samples (PS1-4) and NTC visualized by color change from pink to yellow after 40 min at 65°C. (b) The agarose gel image of the amplified product was obtained from the RT-LAMP reaction. (c) The lateral flow readout results of the *IS6110* gene from MTB culture RNA as a positive control (PC), two negative samples (NS1 and NS2), four positive samples (PS1-4), and NTC.

20 healthy control samples based on the ZN staining, AFB culture, and GeneXpert reports obtained from AIIMS, Bhopal. We confirmed that 157 PS (true positives) tested positive with our CLAP-TB assay, while four samples showed false negatives by CLAP-TB assay, and all the 71 NS (true negatives) tested negative (Fig. 9; Table S1). Further, the TPR, FPR, FNR, and TNR were calculated and found to be 97.51%, 0%, 2.49%, and 100%, respectively. Moreover, for the detection of these 232 clinical samples, the overall clinical sensitivity of the assay was 98.27%.

Discussion

Rapid and user-friendly detection methods for TB diagnosis are still a concern. The current diagnostic methods mainly involve AFB smear microscopy by ZN staining, culture-based methods, and molecular detection (18,19). ZN staining is not only less sensitive but fails to detect MTB from non-tuberculous mycobacteria (NTM). Similarly, culture methods, although they are more sensitive than ZN staining, require long incubation times (2-8 weeks) apart from being non-specific. Advancements in molecular diagnostics are impeded due to high costs and the requirement for infrastructure, which limits their use in less developed nations. Thus, to achieve the TB elimination goal, rapid, sensitive, and user-friendly diagnostics methods are urgently needed and could be deployed, particularly in resource-limiting regions.

In recent times, isothermal amplification techniques such as loop-mediated isothermal amplification (LAMP), recombinase polymerase amplification (RPA), and recombinase-aided amplification (RAA) have emerged as a better alternative to

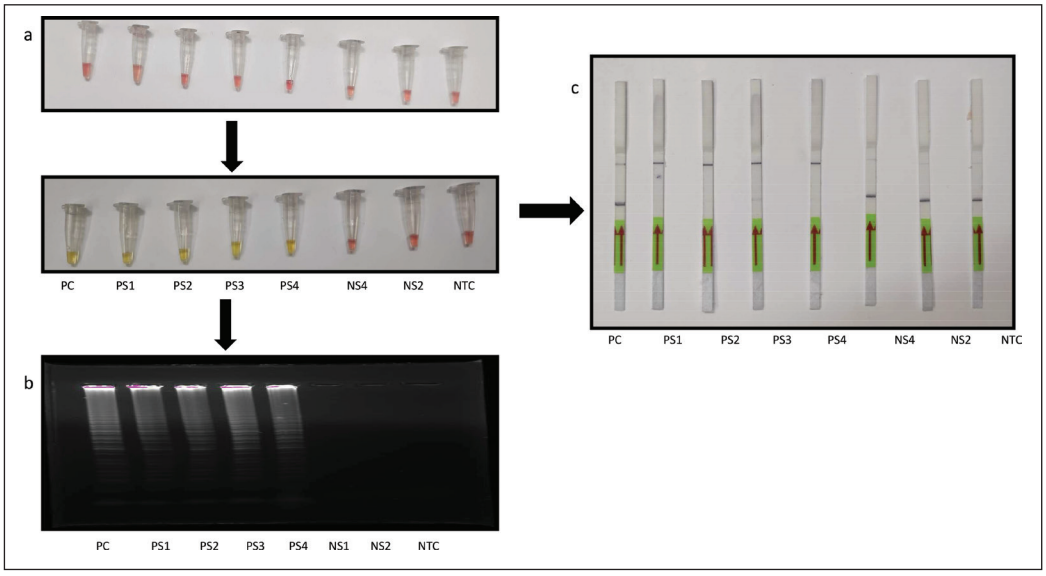

FIGURE 7 - CLAP-TB assay with clinical serum samples (a) Representative RT-LAMP reaction of *IS6110* gene from MTB culture RNA as the positive control (PC), three negative samples (NS1-3), two positive samples (PS1 and PS2) and NTC visualized by color change from pink to yellow after 40 min at 65°C. (b) The agarose gel image of the amplified product was obtained from the RT-LAMP reaction. (c) The lateral flow readout results of the *IS6110* gene from MTB culture RNA as the positive control (PC), two negative samples (NS1-3), two positive samples (PS1 and PS2), and NTC.

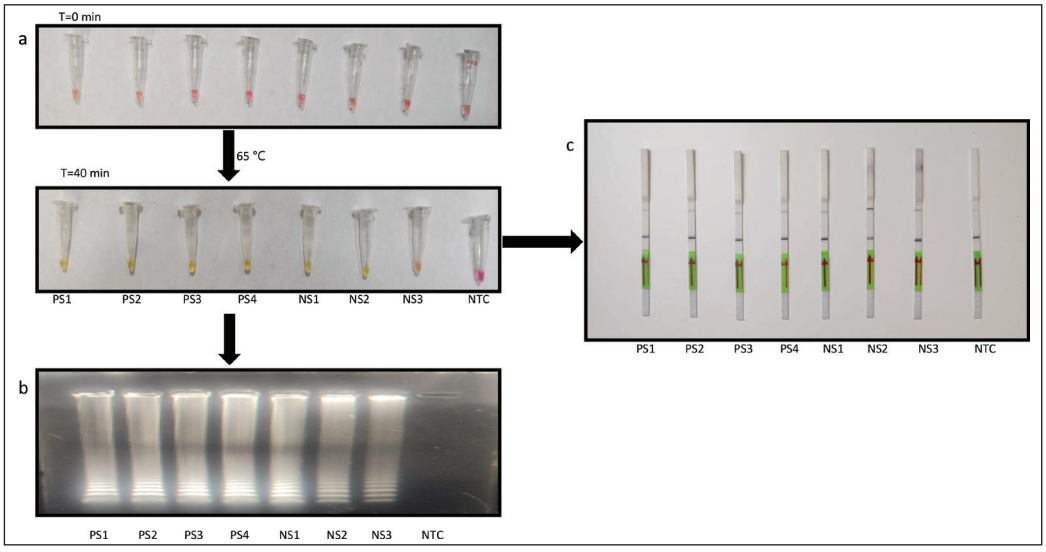

FIGURE 8 - CLAP-TB assay for internal control *POP7* gene (a) Representative RT-LAMP reaction of *IS6110* gene from four positive samples (PS1-4), three negative samples (NS1-3), and NTC visualized by color change from pink to yellow after 40 min at 65°C. (b) The agarose gel image of the amplified product was obtained from the RT-LAMP reaction. (c) The lateral flow readout results of the POP7 gene for four positive samples (PS1-4), three negative samples (NS1-3), and NTC.

Clinical samples (PS)	TPR=TP/(TP+FN)= 157/157+4=97.51%	FNR=FN/(TP+FN) = 4/157+4 =2.49%
Negative samples (NS)	FPR=FP/(FP+TN)= 0/0+71 =0%	TNR=TN/(TN+FP) = 71/71+0 = 100%

FIGURE 9 - Statistical analysis of the performance of the CLAP-TB assay. Statistical analysis of the *IS6110* gene was performed on 232 clinical samples, comprising 161 positive (PS) and 71 negative (NS) samples. True positive rate (TPR) and false negative rate (FNR) are depicted for PS in the upper box, while false positive rate (FPR) and true negative rate (TNR) are depicted for NS in the lower box.

PCR due to their simplicity and sensitivity (20,21). Likewise, recent research has demonstrated the considerable diagnostic potential of CRISPR technology in TB detection. However, in general, CRISPR-based diagnostic methods rely on two steps involving nucleic acid amplification followed by CRISPR-guided sequence detection that confers the specificity attributes (17,19,22-25). LAMP, coupled with CRISPR, has emerged as a promising tool for developing highly sensitive, specific, and user-friendly diagnostic methods (26,27). However, the drawback of the LAMP technique is that it fails to detect viable MTB cells. Hence, the present study offers to overcome this limitation of prior technology and instead developed an RT-LAMP integrated CRISPR-based method for TB diagnosis, which is not only rapid and user-friendly but sensitive and specific, allowing rapid (~134 min) visual TB diagnosis.

Around 25 copies of the *IS6110* gene are present in the MTB H37Rv genome, and due to this abundance, priorities in the detection of MTB (28). Moreover, it is reported to be absent in NTM, thus presenting a specific target (29). Therefore, we selected the *IS6110* gene segment as the target for our CRISPR-Cas coupled RT-LAMP amplification protocol for TB detection (nominated as CLAP-TB assay). Firstly, the CLAP-TB assay was standardized on MTB RNA. Visual detection by color change was confirmed from an RT-LAMP system designed to provide fast and clear visual detection of amplification based on the production of protons and subsequent drop in pH that occurs from the extensive DNA polymerase activity in an RT-LAMP reaction, producing a change in solution color from pink to yellow. The reaction was performed on RNA, followed by gel electrophoresis validation (Figs 1a and b). Then CRISPR-Cas was integrated, and positive results were interpreted by the banding pattern on the lateral flow dipsticks (Fig. 1c). In the present study, we used the Cas 12a enzyme. Additionally, there are different types of Cas9 and Cas 13 enzymes, but the Cas 9 enzyme does not have collateral cleavage activity and is mostly used in gene editing studies. On the other hand, although Cas 13 has collateral cleavage activity but against single-stranded RNA, unlike DNA, Cas 12 hence suffers from RNA stability issues. Moreover, Cas 12 is widely used in different types of bacteria and virus infection (23,30).

Next, we explored the LOD of CLAP-TB assay and found to be 1 ag of RNA per reaction, which agreed with our laterflow readout result (Fig. 2). Previous reports have shown that RT-LAMP is 10 to 100 times more sensitive than conventional PCR assays and other isothermal amplification techniques, e.g., CPA, PSR, or HAD (9). Additionally, the amplification efficiency of RT-LAMP is high with a short reaction time (40 min). A considerable number of LAMP-based studies have been reported on TB diagnosis (31). However, we also compared the sensitivity of CLAP-TB assay using DNA as a template and intriguingly found that using RNA was more sensitive than DNA (Fig. 3). Furthermore, we observed that CLAP-TB assay could also distinguish between MTB and other strains used in the study including two ZN positive bacteria, one Gram-negative bacteria and one human pathogenic fungus where we could detect specific detection of MTB RNA only as apparent from RT-LAMP amplicon color change (Fig. 4a), gel electrophoresis (Fig. 4b) and lateral flow strips band pattern (Fig. 4c).

Based on these results, further, we sought to use the clinical samples (PS/NS) to validate the CLAP-TB assay. For this, we utilized three different types of clinical samples, namely sputum, urine, and serum, where all the samples were isolated from the same patient (Figs 5-7; Table S1). The CRISPR-Cas further confirmed that the true positive clinical samples were tested positive while true negative samples were tested negative by our assay (Figs 5-7). There are reports which suggest that TB can be diagnosed with samples other than sputum, such as urine (32) and serum (33). CLAP-TB can diagnose TB from three different sample types, i.e., sputum, urine, and serum; hence, it opens new avenues for sample collection for the sake of easiness. For instance, urine collection doesn't require any invasive method. Similarly, collecting blood is much easier than sputum collection. Furthermore, CLAP-TB assay detecting MTB from urine may help in the diagnosis of EPTB, which is not easy to detect. Additionally, the isolation of RNA from serum will also aid in overcoming the decontamination step required in the case of sputum and urine.

For further confirmation, a cellular housekeeping gene (internal control) for humans, the *POP7* gene, which encodes for human ribonuclease P and is readily used to detect the presence of cellular materials in patient samples (14,15,34) was utilized. Our CLAP assay confirmed that the color change was observed in both PS and NS contrary to NTC (Figs 8a and b). Similarly, the lateral flow readouts also depicted distinguishable banding patterns as compared to NTC (Fig. 8c). Lastly, the CLAP-TB assay overall showed good correlation with the laboratory results and depicted 98.27% clinical sensitivity and specificity, respectively (Fig. 9; Table S1). The CLAP-TB assay has been worked out with a water bath in the present study; hence, this process does not require an incubator or any heavy equipment. However, to ensure the applicability for the remote areas, the assay has also been standardized in the water kettle or thermos flask (supplementary file; Fig S1 and S2).

Conclusion

Although the proposed CLAP-TB assay may suffer from the drawback of testing only a limited number of clinical samples for this study, nevertheless, the study has demonstrated considerable proof-of-concept that warrants investigation on many clinical samples. Considering the WHO guidelines, where rapid, accurate, and affordable diagnostic assays for TB that do not require sophisticated infrastructure are being investigated in the future, the CLAP-TB assay could be a promising tool serving the purpose in this direction. Moreover, the proposed assay has provided an alternative method to not only detect pulmonary TB but extrapulmonary TB, which is difficult to diagnose.

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