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Development and evaluation of rapid and specific *sdaA* LAMP-LFD assay with Xpert MTB/RIF assay for diagnosis of tuberculosis

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ABSTRACT

There is need for rapid and cost-effective diagnostic test for tuberculosis. The present study was carried out to design a Loop-mediated isothermal amplification (LAMP) assay combined with lateral flow dipstick (LFD) as a point-of-care method for diagnosis of TB. LAMP assay targeting *sdaA* gene combined with LFD for sequence specific detection was standardized in user friendly and rapid format. It does not require sophisticated instruments and shows visual results instantly. The LAMP-LFD assay was validated using culture confirmed specimens. The assay was evaluated in a cross-sectional study using respiratory specimens collected from patients in Delhi, India and it showed high concordance with GeneXpert MTB/RIF assay. Lateral flow dipstick method has provided an excellent detection format with LAMP method. The LAMP-LFD assay showed high diagnostic accuracy in comparison to other methods and can be used as a point-of-care test in cost-effective manner.

1. Introduction

Tuberculosis (TB) has been a global health problem since ancient times. It is the leading cause of death from a single infectious agent for past five years. According to the Global Tuberculosis report by World Health Organization (WHO), 10 million incident cases of TB were estimated in 2017 (WHO, 2018). More than a million lives are lost to the disease annually. The increase of antibiotic resistance in the causative bacterium has posed a threat to the present global TB control measures. However, in more than 85% of the cases, TB is a curable disease if diagnosed and treated properly. In recent reports published by World Health Organization, there has been an emphasis on missed out cases of TB out of the estimated global incidence (WHO, 2018). These cases are either not reported or not diagnosed.

Accurate diagnosis in timely manner is a pillar to the control of any infectious disease along with right treatment and proper follow-up (Keeler et al., 2006). There have been numerous developments in the field of diagnostics since the advent of molecular biotechnology (Pai et al., 2016). Contradicting this progress, the majority of primary health centers continue to rely on more than 130 years old method which is smear microscopy (Pai and Schito, 2015). This method has low sensitivity and specificity for detection of acid-fast bacteria but has an important role in public health due to cost-effectiveness and simplicity of the technique. Culture of *M. tuberculosis* is the gold standard for diagnosis of TB and shows improved sensitivity and specificity over microscopy (George et al., 2011). However, it is time-consuming and requires special containment facilities due to the biology of the pathogen. Moreover, it is available at referral centers and diagnostic laboratories only.

Several molecular diagnostic methods such as nucleic acid amplification techniques have also been applied for the direct detection of mycobacterium in clinical specimens. WHO has recommended GeneXpert MTB/RIF assay which is a real-time PCR based automated platform for rapid diagnosis of TB as well as screening for rifampicin resistance conferring mutations (Lawn and Nicol, 2011). However, this system requires sophisticated laboratory facilities with continuous power supply and cannot withstand high heat and humidity conditions. The cost per assay is expensive in spite of subsidized pricing.

The current diagnostic tools are not suitable for point-of-care (POC) testing for tuberculosis diagnosis (Pai et al., 2016). Lateral flow assay (LFA) based POC devices are amongst the rapidly growing strategies for qualitative and quantitative analysis (Sajid et al., 2015). It has been applied for the detection of cancer markers, microorganisms, toxins, heavy metals and pesticides (Lu et al., 2017; Wang et al., 2012; Wang

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et al., 2009; Zhao et al., 2016). Antigen detection test such as rapid urine LAM (lipoarabinomannan) is a lateral flow assay-based test for diagnosing TB. However, owing to suboptimal sensitivity and specificity, current urinary LAM assays are deemed unsuitable as a general screening or diagnostic test for TB (Lawn and Gupta-Wright, 2016). Nucleic acid LFA is used for the detection of amplicons which can be formed during the polymerase chain reaction (PCR) or any other nucleic acid amplification method (Connelly et al., 2008).

Loop mediated isothermal amplification (LAMP) is a nucleic acid amplification technique which is well suited for operating in a laboratory with minimum infrastructure and is a potential POC diagnostic test for tuberculosis (Notomi et al., 2000). Many studies have demonstrated the applicability of LAMP for the diagnosis of pulmonary tuberculosis (Neonakis et al., 2011). The TB-LAMP method has been endorsed by WHO as a replacement test for smear microscopy for diagnosis of pulmonary TB in intermediate and high TB burden countries (WHO, 2016). TB- LAMP method has been shown to be comparable to Xpert method in culture positive TB cases but there is little of research data to support its utility in culture negative TB cases.

Using LAMP assay, the 190 bp region of *sdaA* (L-Serine dehydratase) gene has been evaluated as a diagnostic target for the diagnosis of tuberculosis using nucleic acid amplification method in our previous studies (Joon et al., 2015; Nimesh et al., 2014). It has shown good sensitivity and specificity making it a promising target for the detection of *M. tuberculosis* in clinical specimens. The problem of carry over contamination due to opening of tubes post amplification has been addressed using Uracil-DNA glycosylase (UNG) treatment (Nimesh et al., 2014). Lateral flow dipstick (LFD) method has provided an excellent detection format for rapid visualization of nucleic acid amplification products and it can be combined with LAMP for sequence specific end point detection (Sajid et al., 2015). In the present study, *sdaA* LAMP assay was combined with LFD for sequence specific detection and its diagnostic accuracy was evaluated in comparison with GeneXpert MTB/RIF assay.

2. Materials and methods

2.1. Specimen collection and processing

2.1.1. Ethics statement

The information sheet and consent form used were approved by the ethical committee of the institution (Dr. BR Ambedkar Center for Biomedical Research, New Delhi; Ethical clearance number F50-2/Eth.Com/ACBR/11/2108 and number F50-2/Eth.Com/ACBR/15/). Informed written consent from all the participants involved in our study has been obtained.

2.1.2. Pulmonary specimens

Sputum specimens ($n = 151$) from the patients of pulmonary tuberculosis, received in the Department of Microbiology, Vallabhbhai Patel Chest Institute (VPCI), Delhi, during the period from October 2015 to March 2016 were collected in accordance with the institute ethical guidelines. Of the samples collected, samples that were insufficient in amount, were of inadequate quality or lacked clinical details of the patients were excluded and 107 were used for further analysis. The basic information such as patient enrolment number, date of sample collection, age, sex, relapse status, acid-fast bacilli (AFB) smear microscopy results and culture results (after 8 weeks) were recorded. The specimens were also subjected to testing by GeneXpert MTB/RIF assay. The sputum specimens left after routine smear microscopy and culture were collected and processed further for DNA extraction. The study results did not affect the decision to administer therapy to the subjects. In addition to these, DNA extracted from the sputum specimens of 18 culture positive TB patients previously used for evaluation of *sdaA* LAMP method were evaluated in the present study to validate the diagnostic potential of the assay (Nimesh et al., 2014).

2.1.3. Inclusion and exclusion criteria

Patients with one or more of the following clinical symptoms: productive cough, low grade fever, blood in sputum, pain, dyspnea, weight loss, general weakness or night sweats were included in the study. Subjects already on anti-tubercular therapy (ATT) at the time of specimen collection were excluded from the study.

2.1.4. Processing of sputum specimens

All the sputum specimens were evaluated using Ziehl Neelsen acid-fast staining for smear microscopy. Solid culture was performed using Lowenstein Jensen (LJ) medium and the culture vials incubated at 37 °C for eight weeks. Evaluation with GeneXpert MTB/RIF assay was carried out according to the manufacturer's guidelines. For *sdaA* PCR and LAMP assay, sputum samples were processed by the Universal Sample Processing (USP) method for DNA extraction (Chakravorty et al., 2005). DNA isolation was performed by addition of Chelex-100 solution and incubating the mixture at 90 °C for forty minutes. The isolated DNA was stored at −20 °C and used for PCR assays and LAMP assays.

2.2. Standardization and evaluation of sdaA LAMP-LFD assay

The reaction conditions used for *sdaA* LAMP assay were the same as described in previous study (Nimesh et al., 2014). The assay was standardized for detection with more specific and easier format using lateral flow dipstick.

The region between F1 and B1c was selected for the designing of a probe for specific detection of amplicon. The sequence was chosen based on general parameters for probe designing i.e. length, secondary structure, GC content, melting temperature. The probe was labelled with FITC (Fluorescein Isothiocyanate) at the 5′ end.

2.2.1. Standardization of detection of sdaA LAMP by lateral flow dipstick

The primer sequences for *sdaA* LAMP assay were kept the same except for an addition of biotin label at the 5′ end of FIP primer sequence. After verifying the successful reaction with biotin labelled primer, various conditions of probe hybridization and detection of lateral flow dipstick were standardized:

When the probe is added to the reaction mixture post amplification, it needs to be hybridized for a brief period of time (5–10 min). The standard conditions were chosen for probe hybridization as reported by various related publications which mention adding 20 picomoles of probe to 25 µl of reaction mix and hybridization at 65 °C for 5 min followed by addition of 10 µl of hybridized product to 100 µl of assay buffer (supplied with LFD) (Kaewphinit et al., 2013; Prompamorn et al., 2011; Thongkao et al., 2015). The effect of temperature on detection line intensity was studied by hybridising the probe at temperatures 61 °C, 63 °C, 65 °C and 67 °C. Alternatively, the probe was added in the reaction mixture of LAMP and analysed using LFD. Various ratios of the hybridized product and assay buffer (supplied with LFD) were tried for detection on LFD. These were 1:4, 1:5, 1:8 and 1:10. The concentration of probe added in the reaction mix post amplification was varied from 10 µM to 40 µM. The probe was hybridized at 65 °C for 5 min followed by addition of 20 µl of the hybridized product to 100 µl of assay buffer and the strip was checked for development of lines after immersing for 5 min.

2.2.2. Analytical sensitivity and analytical specificity of the sdaA LAMP-LFD assay

Serial dilutions of the genomic DNA (5 fg to 1 ng) of *M. tuberculosis* H37Rv strain were tested in triplicate to check the analytical sensitivity of the LAMP-LFD assay. The genomic DNA was isolated from solid culture of *M. tuberculosis* H37Rv strain and its concentration was calculated by measuring absorption at 260 nm using NanoDrop spectrophotometer (Thermo Scientific) and concentration of DNA was adjusted with sterile/autoclaved water to 1 ng/µl. It was further used to make ten-times serial dilutions (with an exception of 5 fg). One microliter of

each dilution (5 fg to 1 ng) was used as template for each LAMP reaction. The hybridized product was mixed with assay buffer and LFD was immersed for five minutes. Lowest limit of detection for other end-result detection formats was also compared.

To confirm the specificity, *sdaA* LAMP-LFD assay was performed using one nanogram of genomic DNA isolated from fourteen mycobacterial reference strains procured from the national repository (National JALMA Institute of Leprosy and other Mycobacterial Diseases, Agra, India).

2.2.3. Clinical evaluation of sdaA LAMP-LFD assay

The diagnostic potential of the assay was validated using sputum specimens from suspected patients of pulmonary tuberculosis who visited V.P. Chest Institute, Delhi University, Delhi. The test results were compared with culture, smear microscopy as well as WHO recommended GeneXpert assay. The diagnostic potential of the assay was evaluated using 18 specimens from culture confirmed TB patients. In 107 specimens, the sensitivity and specificity of *sdaA* LAMP-LFD assay was determined in comparison with GeneXpert assay.

3. Results

The *sdaA* LAMP assay was standardized for detection with more specific and easy detection format using lateral flow dipstick. The universal generic LFD was used which can detect analyte labelled with biotin and FITC. The sequence of the probe labelled with FITC is as follows: 5′ AACCCTCCGCCCACCG 3′. Based on the intensity of test line (as indicated by visual inspection), it was concluded that the maximum hybridization was achieved at 65 °C. It was also observed that the probe added in the reaction mixture for LAMP yields similar results in development of test line on LFD. It was observed that the1:5 ratio of hybridized LAMP product to assay buffer gave optimal intensity test line as well as control line. It was observed that an increase in intensity of test line correlated with increase in probe concentration. It was concluded that 20 µM of probe concentration should be added for optimal detection on LFD.

The *sdaA* LAMP-LFD assay was able to detect up to 5 fg of purified DNA of *M. tuberculosis* which theoretically corresponds to 1–1.3 copy of *M. tuberculosis* genome (Fig. 1). For determination of analytical specificity of the assay, the test line was observed when the genomic DNA isolated from *M. tuberculosis* H37Rv was used as template for the *sdaA* LAMP assay (Fig. 2). In addition, test line bands were not observed when genomic DNA from 12 other species of Mycobacterium was used as template. However, a test band was observable with *M. bovis* (AN-5) DNA. This DNA had been isolated from a pathogenic strain which is included in *M. tuberculosis* complex.

The optimized conditions were used for further evaluation of clinical specimens for detection of *M. tuberculosis* with *sdaA* LAMP-LFD assay. The *sdaA* LAMP assay was evaluated using purified genomic DNA isolated from the sputum specimens of 18 culture confirmed TB cases. All the specimens showed positive results with the test assay as indicated by the development of test lines on LFD.

To ascertain the diagnostic accuracy of the test in comparison with WHO recommended molecular method GeneXpert MTB/RIF assay, sputum specimens were collected from VPCI, Delhi and evaluated using various methods. Out of 151 specimens, 107 were included to evaluate the diagnostic potential of *sdaA* LAMP-LFD assay (Fig. 3). The detailed results of specimens with all methods have been presented in Table 1. The amplified products in positive specimens were visualized by a change to green colour and the development of test line on LFD. Out of the 107 specimens, 15 were positive with GeneXpert assay with one showing rifampicin resistance. These specimens also tested positive with LAMP-LFD assay. Only two specimens were culture positive and smear positive while the other 13 were culture and smear negative. One specimen tested negative with *sdaA* PCR assay but gave positive result by LAMP method as well as LAMP-LFD (Table 1). The statistical analysis of test results showed 100% concordance between test method and GeneXpert assay with Cohen's kappa coefficient value 1.0 as a measure of inter-rater agreement.

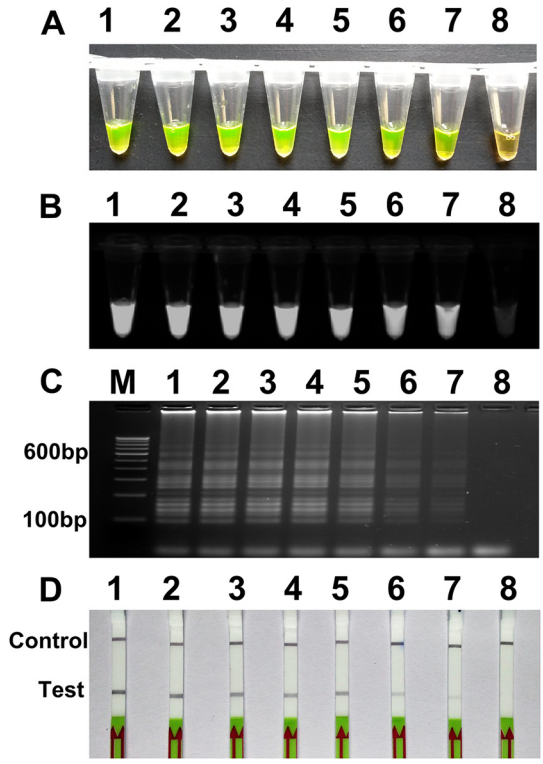

Fig. 1. LAMP reaction results visualized on LFD using purified genomic DNA of *M. tuberculosis* as template. Lanes/tubes 1–7 show LAMP reaction with 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg and 5 fg of genomic DNA as template; lane 8 shows no template control (NTC). A shows visual method using SYBR Green I, B shows detection on UV trans-illuminator, C shows agarose gel electrophoresis and D shows detection limit using lateral flow dipstick. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

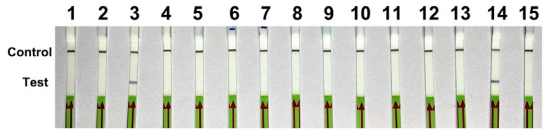

Fig. 2. Visualization of LAMP reaction results on lateral flow dipstick with one nanogram of genomic DNA isolated from the fourteen mycobacterial reference strains as template. Lane 1 is NTC (no template control). Lanes 2–15 show LAMP reaction with purified genomic DNA of *M. avium, M. bovis* (AN-5)*, M. chelonae, M. fortuitum, M. gordonae, M.kansasii, M. marinum, M. scrofulaceum, M. simiae, M. smegmatis, M. szulgai, M. terrae, M. tuberculosis* H37Rv and *M. xenopi,* respectively.

4. Discussion

Diagnostic testing and in particular early detection are very critical for the control of tuberculosis which is one of the deadliest infectious diseases. In the present study, an effort has been made to develop and standardize *sdaA* LAMP assay combined with lateral flow dipstick (LFD) and evaluate its performance for the diagnosis of tuberculosis in pulmonary clinical specimens. *sdaA* LAMP assay was previously evaluated in our laboratory for the diagnosis of pulmonary tuberculosis as well as extra pulmonary tuberculosis using detection by SYBR Green I dye and agarose gel electrophoresis (Joon et al., 2015; Nimesh et al., 2014). The method showed high specificity (97.2%) and sensitivity (100%) in

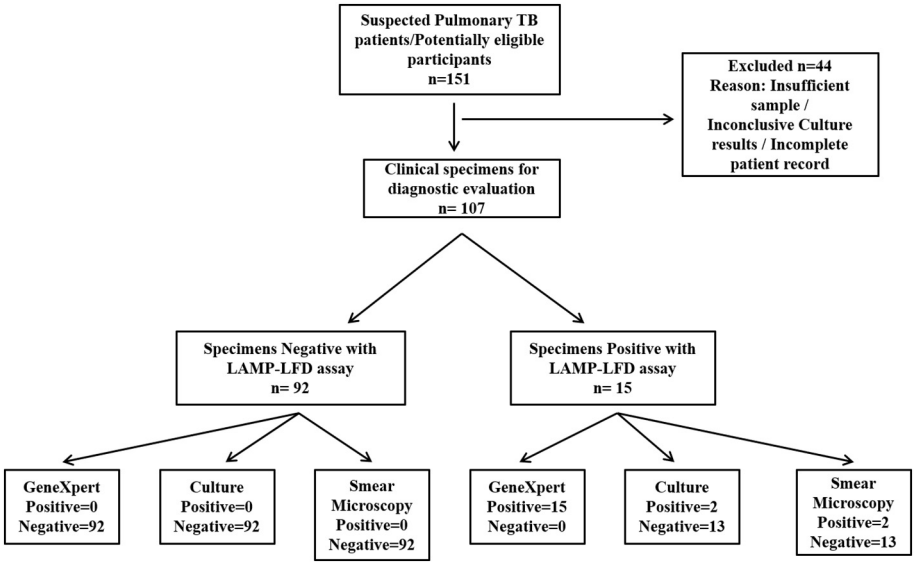

Fig. 3. Flow chart for study according to STARD guidelines.

comparison to culture as gold standard for diagnosis of pulmonary TB (Nimesh et al., 2014).

Application of nucleic acid amplification in point-of-care format requires an easy and simple method for detection of amplification. An ideal detection format must provide an additional specificity and sensitivity without causing a significant increase in the test costs. The common detection formats for LAMP assay are expensive or require trained persons for handling. Quantitative detection formats such as real time instruments and turbidity meter add to the cost of the assay and require laboratory facilities (Mori et al., 2004). There is a lack of specificity along with ambiguous results using dyes such as SYBR Green I, Calcein or hydroxyl napthol blue which allow direct visual detection of amplified products (Goto et al., 2009; Tomita et al., 2008). These limitations make them unsuitable for point-of-care use. Lateral flow dipstick is an excellent alternative for the rapid detection of amplification results in a sequence specific manner. This format does not require electricity or instrument. The combined use of LAMP and LFD can lead to a sensitive, specific and cost-effective diagnosis of TB in low resource settings and remote areas.

Detection of LAMP products was carried out using generic lateral flow dipstick. The optimum temperature of hybridization was the same as isothermal amplification which overcomes the need to maintain different temperatures. The optimized conditions for analysis using LFD were similar to previously reported studies for LAMP combined with lateral flow dipstick (Huang et al., 2017; Prompamorn et al., 2011; Rigano et al., 2014; Yongkiettrakul et al., 2014). Furthermore, LAMP-LFD assay can also be carried out by addition of labelled probe as loop primer in the reaction mix for simultaneous amplification and hybridization during LAMP amplification step. There was no adverse effect on the amplification efficiency and this can reduce the chance of contamination and operation time.

The LAMP-LFD assay showed excellent analytical sensitivity by detecting as low as 5 fg of genomic DNA of *M. tuberculosis*. There are few reports of increase in limit of detection by using generic LFD while several studies have demonstrated same analytical sensitivity as other methods (Huang et al., 2017; Kaewphinit et al., 2013; Thongkao et al., 2015; Yongkiettrakul et al., 2014). The analytical specificity of LAMP-LFD was also evaluated and our assay showed positive results only with species of MTBC which validated the specificity of the method.

The clinical evaluation of *sdaA* LAMP-LFD assay was carried out for the diagnosis of pulmonary tuberculosis. The assay was applied for the detection of culture confirmed TB in 18 specimens and all the specimens tested positive proving the high sensitivity and reliability of the method. Many other LAMP assays for detection of MTB have shown 100% sensitivity. Various LAMP assays have shown good performance for diagnosis of smear positive and culture positive TB. The main challenge is the diagnosis of smear negative and culture negative TB where most of NAAT methods perform poorly. Therefore, to assess the diagnostic potential of *sdaA* LAMP assay for diagnosis of pulmonary TB, it was evaluated in comparison with WHO recommended GeneXpert

Table 1
Details of positive specimens for diagnosis of pulmonary tuberculosis by smear, culture, *sdaA* PCR, LAMP-LFD and GeneXpert MTB/RIF methods.

Specimen No.	Smear	Culture	*sdaA* PCR	LAMP-LFD	GeneXpert	Rifampicin resistance
4	Positive	Positive	Positive	Positive	MTB detected	Yes
6	Positive	Positive	Positive	Positive	MTB detected	No
13	Negative	Negative	Positive	Positive	MTB detected	No
26	Negative	Negative	Positive	Positive	MTB detected	No
28	Negative	Negative	Positive	Positive	MTB detected	No
31	Negative	Negative	Positive	Positive	MTB detected	No
41	Negative	Negative	Positive	Positive	MTB detected	No
52	Negative	Negative	Positive	Positive	MTB detected	No
58	Negative	Negative	Positive	Positive	MTB detected	No
71	Negative	Negative	Negative	Positive	MTB detected	No
72	Negative	Negative	Positive	Positive	MTB detected	No
79	Negative	Negative	Positive	Positive	MTB detected	No
83	Negative	Negative	Positive	Positive	MTB detected	No
91	Negative	Negative	Positive	Positive	MTB detected	No
94	Negative	Negative	Positive	Positive	MTB detected	No

MTB/RIF assay. The GeneXpert MTB/RIF test had shown a fair sensitivity and specificity for diagnosing smear negative pulmonary TB (Reechaipichitkul et al., 2016; Tadesse et al., 2016).

In this study, 107 specimens were evaluated by various methods such as smear microscopy, solid culture, GeneXpert MTB/RIF assay, *sdaA* PCR assay and LAMP-LFD assay. *sdaA* LAMP assay showed high diagnostic accuracy by correctly detecting all the cases of TB which were smear and culture negative but positive with GeneXpert MTB/RIF assay. These patients were treated with anti-tubercular therapy based on GeneXpert results. The sensitivity and specificity of test in our study are higher than summary estimates of sensitivity of 89.6% (95% CI 85.6–92.6%) and specificity of 94% (95% CI 91–96.1%) reported in recent meta-analysis of diagnostic accuracy of the LAMP assay for culture confirmed tuberculosis which included 26 studies on TB-LAMP and various in-house LAMP assay (Nagai et al., 2016). However, it is well within the range of sensitivity and specificity reported by studies evaluating in-house LAMP assay (Nagai et al., 2016). The present study has higher positive rate in smear negative/culture negative specimens. It may be due to the reason that all the specimens are not tested with Xpert MTB/RIF method owing to high cost and limited supply of these cartridges. The sensitivity and specificity cannot be ascertained in smear and culture negative specimens due to lack of perfect gold standard for comparison. However, the results were confirmed in these cases by clinical follow-up and all the patients responded to anti-tubercular therapy based on Xpert MTB/RIF assay results.

This study determined the diagnostic potential of *sdaA* LAMP-LFD assay in comparison with GeneXpert system which requires more infrastructure and expensive instrumentation. This method is simple, rapid, convenient, sensitive, specific and an affordable alternative to real-time nucleic acid amplification technologies because the assay can be carried out isothermally even on battery powered device or heat bath and the results can be visualized by the naked eyes which makes it suitable and feasible for detection even at low resource settings.

There are some important considerations to make this assay more suitable for point-of-care application. The method used for processing sputum specimens for DNA isolation needs to be simplified further although USP methodology used in this study had been proposed to be carried out at primary health care settings. The need for requirement of refrigerated cold chain also impairs the usefulness of nucleic acid amplification test in many low resource settings. Stability of test reagents and shelf-life in ambient storage conditions should be the characteristics of a point-of-care test. It has been reported by few publications that LAMP assay reagents can be dried for storage and distribution under ambient conditions (Hayashida et al., 2015; Hayashida et al., 2017; Yoshikawa et al., 2014). As preliminary work, we have tested *sdaA* LAMP-LFD assay with lyophilized reagents and it showed positive results.

In conclusion, the combination of LFD with *sdaA* LAMP assay makes it a suitable diagnostic tool for resource limited settings which lack sophisticated and expensive instruments. The simplicity of lateral flow dipstick and LAMP assay offers potential for the development of a molecular diagnostic test which can be used for diagnosis of TB at point-of-care settings. The outcome of this assay can be used in adjunct to smear microscopy at primary healthcare settings.

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