



Application of loop mediated isothermal amplification (LAMP) assay as an alternative diagnostic test for rapid tuberculosis diagnosis in limited resource setting

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ABSTRACT

Diagnostic potentiality of loop mediated isothermal amplification (LAMP) assay with human sputum samples was compared with smear microscopy, culture and PCR in providing rapid and accurate TB diagnosis. Practical application of this assay was emphasized as a supplementary diagnostic test with acid fast smear microscopy for controlling tuberculosis in limited resource setting particularly India. Sputum samples (261) from TB suspected patients were collected, processed and subjected to smear microscopy, culture, PCR and LAMP assay. These 4 diagnostic methodologies were compared in terms of sensitivity, specificity, and rapidity and user friendliness. Sensitivity of LAMP, PCR and microscopy were 97.47, 97.47, and 61.61%, respectively, and specificity of LAMP, PCR and microscopy were 60.31, 74.60 and 100% respectively. Higher sensitivity of diagnostic test will be more useful in high TB endemicity area to cover most of TB patients under National Tuberculosis Control Programmes. This LAMP assay may be a viable alternative of costly PCR diagnosis. This test may be adopted as supplementary diagnostic test with acid fast smear microscopy for routine diagnosis of human TB.

Key words: *Mycobacterium tuberculosis*, LAMP, TB diagnosis

Tuberculosis has been posing a serious challenge to public health globally. Approximately 9.4 million new tuberculosis cases annually are reported worldwide out of which 2 million cases are from India. India is having highest TB burden accounting for one-fifth (21%) of the global incidence (WHO global TB report 2010). Early tuberculosis diagnosis is prerequisite for effective treatment and control of tuberculosis. Acid fast smear microscopy is mainstay for TB diagnosis at primary health care centre in India. TB diagnosis based on ZN staining has several limitations like low sensitivity, unable to differentiate *Mycobacterium tuberculosis* complex bacteria with non tuberculosis *Mycobacteria* organism, false negative due to low number of bacilli in sputum and need of multiple visits by patients to provide clinical specimens has lead to missing of several tuberculosis positive cases. Nucleic acid based diagnostic tools for tuberculosis are undoubtedly rapid, sensitive but high cost, use of sophisticated equipment, need of skilled technician and complex result analysis make these impractical solution in low resource setting.

Loop-mediated isothermal amplification (LAMP) based assays are particularly relevant for resource limited settings (Neonakis *et al.* 2008, Lange and Mori 2010). Some of these

assays have been used with clinical specimens (Pandey *et al.* 2008, Poudel *et al.* 2009, Zhu *et al.* 2009, Aryan *et al.* 2010). LAMP has several advantages as compared to the other molecular diagnostic methods like LAMP reaction can be performed at isothermal condition (60–65°C) in a water bath obviating need of costly thermocycler machine, high sensitivity and specificity due to using 6 primers targeting 4 distinct region of a gene. In addition to that result interpretation can be made by just visual detection of turbidity and color change after addition of syber green dye, make this assay system user friendly.

MATERIAL AND METHODS

Mycobacterial isolates (51) comprising both MTBC and NTM maintained at *Mycobacteria* Laboratory, IVRI, Izatnagar (*M. tuberculosis*: 20, *M. bovis*: 20, *M. bovis* BCG: 3, *M. caneti*: 2, *M. microti*: 2, *M. phlei*: 2, *M. fortuitum*: 1, *M. avium*: 2). All the mycobacterial isolates were grown in Lowenstein Jensen (LJ) - glycerol and LJ- pyruvate medium at 37°C for 6–8 weeks. These mycobacterial isolates were further confirmed by ZN staining, battery of biochemical tests (Verma and Srivastava 2001) and MTBC complex specific PCR (Thangaselvam *et al.* 2009).

Sputum samples (261) were collected from TB suspected patients at District Tuberculosis Hospital, Bareilly, Uttar

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Pradesh, India. Approximately 5 ml mucoid or muco-purulent early morning sputum sample was collected from patients having history of frequent coughing, dyspnea, pathology in chest X-ray radiograph or already undergoing DOTs treatment. Immediately after collection all sputum samples were subjected to smear microscopy and processing was done by modified Petroff's method (Salem *et al.* 2007). Sediment of processed sample was re-suspended in 2 ml of phosphate buffered saline (PBS) and 0.5 ml was inoculated into freshly prepared LJ medium with 1% glycerol and LJ medium with 0.5% pyruvate, incubated at 37°C for 6–8 weeks. Cultures were examined weekly for any contaminated growth. Genomic DNA was extracted from all the processed sputum samples by commercial genomic DNA extraction kit following instruction supplied by manufacturer. Genomic DNA was extracted from *M.tuberculosis* H37Rv and *M.phlei* for positive control and negative control respectively by boiling method (Mazars *et al.*, 2001) and CTAB method (Rodríguez *et al.* 1997). Briefly in boiling method, 2 loopful growth of mycobacterial culture in 400 µl of 1X TE buffer, boiled at 100°C for 10 min, followed by snap chilling in ice. The procedure was repeated 3 times, centrifuged at 16500 g (12,000 rpm, Hermle refrigerated centrifuge) for 5 min and supernatant was used as DNA template and stored at –20°C till further use.

In-house LAMP components and reaction length with IS6110 primer was optimized. Different combinations of reaction component were applied to reduce reaction time with easy result interpretation obviating any sophisticated equipment and technical skills. Further LAMP results were confirmed by restriction enzyme digestion with *MlsI* restriction endonuclease. Specificity was examined with available NTM organism (*M.Phlei*, *M.fortuitum*, *M.avium*) and other common contaminant bacteria (*E.coli*, *S.pyogenes*, *S.aureus*, *Parueginosa*).

All 261 genomic DNA templates from clinical specimen were subjected to PCR assay (Thangaselvam *et al.* 2009) and LAMP assay (Aryan *et al.* 2010) in duplicate with positive and negative control simultaneously. Briefly, PCR was carried out in 25 µl volume, using 200 µM dNTPs, 2.5 µl 10X PCR buffer, 10 picomole of each primer, 0.7 mM of MgCl₂, 1U of *Taq* polymerase and 5 µl of 10 ng/µl the template DNA. PCR cycling conditions were initial denaturation at 94°C for 10 min, followed by 30 cycles at 94°C for 1 min, 56°C for one minute, 72°C for 1 min and a final extension at 72°C for 10 min. The amplified products were subjected to electrophoresis on a 1% agarose gel containing ethidium bromide. Briefly, LAMP reactions were performed in a total volume of 25 µl consisting of 30 pmol each of inner primers FIP and BIP, 5 pmol each of outer primers F3 and B3, 20 pmol each of loop primers FLP and BLP, 2.4 mM deoxynucleoside triphosphate, 0.6 M betaine, 2.5 mM MgSO₄, 1x buffer (25 mM Tris- HCl pH 8.8, 12.5 mM KCl, 12 mM (NH₄)₂SO₄ and 0.1% Tween 20), 8 U *Bst* DNA polymerase and 2 µl of

10 ng/µl DNA template. LAMP results were considered positive as turbidity or color change after addition of SYBR green dye and ladder like DNA electrophoretic pattern.

RESULTS AND DISCUSSION

Approximately 9.4 million new cases of tuberculosis annually (TB) recorded worldwide and around 2 million deaths occur every year associated with TB (WHO report 2010). The major proportion of these cases and deaths occur in developing countries like India having poor infrastructural facilities where the diagnosis of TB is based primarily on the examination of sputum smears for acid-fast bacilli. Controlling TB in India is a tremendous challenge. (<http://www.tbindia.org/rntcp.asp>). This may be attributed to poor socio-economic condition, limited diagnostic facilities, low sensitive diagnostic test, under compliance of DOT's strategy and emergence of MDR and XDR tubercle bacilli.

Early, accurate and rapid identification of positive tuberculosis case is necessary for controlling tuberculosis. The standard laboratory diagnosis of TB relies on the detection of acid-fast bacilli in smear microscopy and on the culture of the organism on appropriate media. In acid fast microscopy ZN staining is done directly with mucopurulent sputum sample for 3 consecutive days and positive specimen is graded as +3, +2, +1 as per RNTCP guidelines. High drop out of positive TB cases is one of the most troublesome problems in India due to relying on smear microscopy results only and multiple visit of patients to provide clinical specimen also potentiate this problem (Shen *et al.* 2009, World Health Organization 2006). Isolation of Mycobacterium organism from clinical specimen is considered as a gold standard but certain limitations are associated with conventional diagnostic test such as—it is very tedious procedure, takes 6–8 week time, technical skill demanding and high contamination rate due to delayed processing or faulty decontamination procedure.

Antibody based diagnostic tools for TB diagnosis are not so much successful due to irregular humoral immune response in tuberculosis, low sensitivity, cross reactivity with other NTM bacteria, high cost, erroneous interpretation (Steingart *et al.* 2007). Genotypic tests for identification of mycobacterial species are now considered as novel approach in diagnostic mycobacteriology. Molecular tests are able to provide a simple, rapid and more accurate detection of *M. tuberculosis* in clinical samples (Cho 2007). However, the high cost of molecular detection methods such as polymerase chain reaction (PCR), where specialized equipment and reagents are required (Chakravorty *et al.* 2005, Chakravorty and Tyagi 2005) limits their application in developing countries. Thus, there is a need to find alternative and economically viable diagnostic methods applicable in the field. Loop mediated isothermal amplification technique is a novel approach to develop highly sensitive nucleic acid based diagnostic test. LAMP method has been successfully

applied for the detection of bacteria, virus, fungi, parasites, mycoplasma and genetic modified organisms. It has been used in detection of *Escherichia coli* O157:H7 (Maruyama *et al.* 2003), MTB complex, *M. avium*, and *M. intracellulare* (Iwamoto *et al.* 2003), Japanese yam mosaic virus (Fukuta *et al.* 2003), H5N1 avian influenza virus (Imai *et al.* 2006).

In this study, LAMP assay using published primers (Aryan *et al.* 2010) were used to determine practical utility and feasibility in diagnosing human tuberculosis in limited resource setting. LAMP component, temperature and reaction length were in-house optimized and modified for enhancing practical utility of assay. For evaluation of LAMP assay, 261 sputum samples were subjected to smear microscopy, culture, PCR and LAMP assay. Among the 261 suspected cases of tuberculosis, 223 (85.44%), 214 (81.99%), 122 (46.74%), 198 (75.86%) were found positive with LAMP, PCR, ZN staining microscopy and culture respectively. Five cases were detected false positive with smear microscopy and bacterial cultures positive belonging to non tuberculosis mycobacteria and further confirmed by bio-chemical test and molecular diagnostic test. PCR detected 92 (35.24%) and 16 (6.13%) additional TB cases as comparing to acid fast smear microscopy and culture respectively. LAMP assay was able to detect 111 (36.78%), 25 (9.57%), 9 (3.44%) additional TB cases which were not detected by acid fast smear microscopy, culture and PCR respectively (Table 1). Relative sensitivity and specificity of acid fast smear microscopy, PCR and LAMP were (61.61, 100%), (97.47, 74.60%), (97.47, 60.31%), respectively, considering bacterial culture as true positive. None of the smear microscopy positive sample turned out negative in bacterial culture.

Low sensitivity of acid fast smear microscopy is biggest limiting factor in TB diagnosis in India where it is primary screening test to start treatment regime. This low sensitivity may be attributed to low number of bacilli in human sputum sample, asymptomatic TB carrier patients and some samples drawn from patients already undergoing DOTs treatment leads to no or less secretion of tubercle bacilli. Undoubtedly some false negative due to human error cannot be ruled out although approximately 80–100 microscopic fields were examined extensively before declaring any sample negative for acid fast bacilli. Specificity of acid fast smear microscopy was 100% considering bacterial culture as true positive but

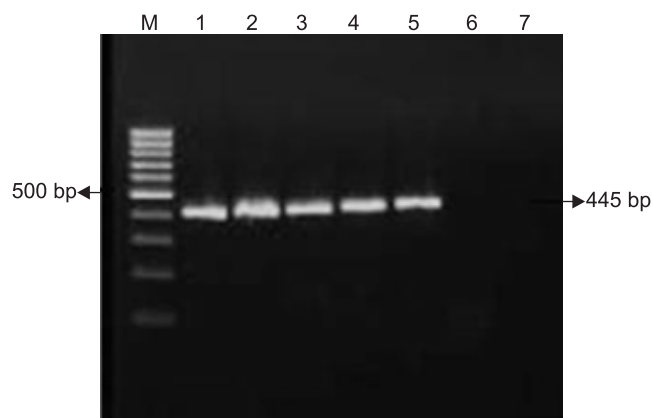
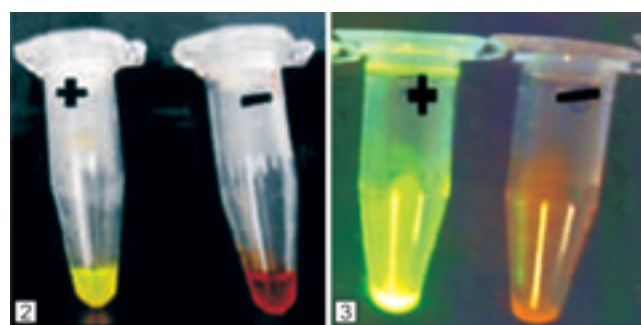


Fig. 1. PCR amplification of MTB complex specific IS6110 region with clinical specimens. M: 100bp DNA ladder, 1–5 positive samples by PCR, 6&7: Negative control.



Figs 2–3. Visualization of LAMP result in day light and U.V. light.

in India where TB is endemic and DOTs programme is being implemented to control tuberculosis, specificity is comparatively less concerned. Approximately 29% acid fast smear negative cases were detected in bacterial culture which clearly define shortcoming of smear microscopy. Bacterial culture is considered as a gold standard for disease diagnosis but it is time consuming, tedious procedure requiring decontaminating and processing of sputum samples makes this method unsuitable for TB diagnosis at primary TB diagnostic units. Bacterial culture is not able to differentiate MTBC complex with other NTM bacteria requiring further molecular diagnostic test. Some false negative culture may not be ruled out during decontamination procedure causing

Table 1. Comparative diagnostic potential of LAMP, PCR and microscopy

Standard culture+	LAMP		PCR		Microscopy		Total
	+	–	+	–	+	–	
Culture -	193	5	193	5	122	76	198
Total	25	38	16	47	-	63	63
	218	43	209	52	122	139	261
Sensitivity(%)	97.47	97.47	61.61				
Specificity(%)	60.31	74.60	100				

destruction of viable bacilli. PCR and LAMP are equally sensitive (97.47%) in TB diagnosis with clinical samples but LAMP sensitivity with extracted *M. tuberculosis* H37Rv genomic DNA is 100 times (0.1pg) higher than PCR (1pg). This finding co-relates with previous finding where comparative studies of LAMP assay with conventional PCR have shown the sensitivity of LAMP as 10 to 100 times higher than PCR (Jin Zhang *et al.* 2010, Qu *et al.* 2010). In contrast to our study, other LAMP-based assays targeting *gyrB* (Iwamoto *et al.* 2003, Boehme *et al.* 2007) and *rrs* (Pandey *et al.* 2008) were used for detection of MTB. Sensitivity of IS6110 targeted LAMP assay was almost equal to *rrs* based LAMP assay (97 and 91%) by comparative analysis with microscopy and culture methods to detect *M. tuberculosis* in sputum samples (Pandey *et al.* 2008). This LAMP assay was found specific amplifying only MTBC genomic DNA while no amplification was observed with other related NTM bacteria and other common contaminant bacteria in clinical specimen. This underline practical utility of this assay in detection of mycobacterium organism. These extra TB positive patients revealed by IS6110 targeted LAMP assay may be attributed to high sensitivity, identification of noncultivable bacteria from asymptomatic TB carrier patients. Possibility of few false LAMP positive may not be ruled out due to cross contamination of samples in spite of strict compliance of standard microbiological practices.

IS6110- LAMP assay is rapid, sensitive, cost effective and users friendly diagnostic techniques for identification of MTBC infections from clinical specimens. Although PCR is more specific than LAMP assay but in this study sensitivity of diagnostic test was more emphasized to cover maximum possible TB patients under DOTs umbrella. High sensitivity of LAMP and PCR may help in detection of TB organism from asymptomatic patients, usually smear microscopy and culture negative. These molecular diagnostic tools have much importance in TB diagnosis because of inherent clumping nature of mycobacterium organism causes uneven distribution of organism in clinical samples and some cases may be missed in smear microscopy and culture. These inapparent TB carrier (smear and culture negative) cases are not covered in DOTs under RNTCP (Chakravorty *et al.* 2005). These carrier patients transmit infection to other susceptible human being. These undiagnosed TB cases are real threats in control of tuberculosis in TB endemic region like India. This causes impediment in TB control programme and achieving millennium development goal reducing TB incidence rate and halves prevalence and mortality due to tuberculosis.

This LAMP assay may be useful particularly in TB endemic region like India where highly sensitive diagnostic test is needed to identify false smear negative TB patients. This LAMP assay may be a viable alternative of costly PCR diagnosis and can be used as supplementary diagnostic test with acid fast smear microscopy for routine diagnosis of

human TB in primary DOTs centre. However, further studies are required encompassing large sample size, samples from different stages of tuberculosis patient for determining diagnostic potentiality of LAMP assay in tuberculosis diagnosis and further development of *M. tuberculosis* species specific LAMP assay is needed to strengthen diagnostic arsenal for combating TB in developing countries.

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