



Diagnostic accuracy of loop-mediated isothermal amplification assay for extra-pulmonary tuberculosis in Indian population

Roopali Rajput^a, Paras Singh^{a,*}, Rohit Sarin^b, Prabhpreet Sethi^c, Sangeeta Sharma^d

^a Department of Molecular Medicine, National Institute of Tuberculosis and Respiratory Diseases, Sri Aurobindo Marg, New Delhi, India

^b Department of TB and Respiratory Diseases and Director, National Institute of Tuberculosis and Respiratory Diseases, Sri Aurobindo Marg, New Delhi, India

^c Department of TB and Respiratory Diseases, National Institute of Tuberculosis and Respiratory Diseases, Sri Aurobindo Marg, New Delhi, India

^d Department of Paediatrics, National Institute of Tuberculosis and Respiratory Diseases, Sri Aurobindo Marg, New Delhi, India

ARTICLE INFO

Keywords:

LAMP assay
Molecular diagnosis
Accuracy
EPTB
Children
PCR
Nested
Pediatric

ABSTRACT

Background: Confirmatory diagnosis of extra-pulmonary tuberculosis remains a true challenge owing to difficulty in procuring appropriate specimen, inefficient laboratory methods and paucibacillary nature of infection. These obstructions become all the very difficult in pediatric EPTB cases, due to non-specific clinical signs and symptoms, low sensitivity of smear microscopy and culture, lack of awareness among clinicians, etc.

Aim of the study: The present study aimed to evaluate the diagnostic accuracy of rapid and cost-effective loop-mediated isothermal amplification (LAMP) assay for EPTB diagnosis in children.

Methods: A total of 154 cases were analyzed by EPTB-site smear microscopy, culture, PCRs for IS6110, MPB64 & Pab genes, nested PCR and LAMP assay. Single-gene PCRs were performed by custom-synthesized primers. Nested PCR was performed using the 3B BIOTUB Kit and the LAMP assay was done using the Nu-LAMP TB kit.

Results: We observed that the molecular tests displayed 4-fold higher positivity rate (minimum 46%) in comparison to the microbiological tests (maximum 11.03%). In contrast to the composite reference standard, LAMP assay was found to be 79.6% sensitive and 78% specific for EPTB diagnosis in childhood cases.

Conclusions: Our results indicate that LAMP assay is a promising technique for efficient diagnosis of EPTB in children belonging to resource-limited regions.

1. Introduction

As per the statistics by World Health Organization, 10.4 million TB cases were estimated globally, of which at least a million were in children below 15 years of age (WHO, 2018). The low- and middle-income countries account for over 95% of TB-related deaths, with India leading the mortality rate among the developing countries (WHO, 2017). Approximately 75% of the global pediatric TB cases each year originate from the 22 high-burden countries (Nelson and Wells, 2004). Childhood TB is given low priority in many countries and given the challenges associated with the diagnosis, a true estimate of the pediatric TB burden seems difficult ultimately causing unwanted obstructions toward containing the TB epidemic in children (Newton et al., 2008; Ahmed et al., 2008).

Present methods for diagnosis of TB in children pose several challenges in accurate identification of the disease. Smear microscopy is rapid and easy to perform but has low sensitivity. The gold-standard

culture (solid or liquid) methods are tremendously slow and require well-trained personnel for handling cultures and expensive laboratory equipments. Moreover, both these techniques generate low yields in pediatric extra-pulmonary tuberculosis (EPTB) cases. Chest X-rays provide non-specific findings (Togun et al., 2017) and tuberculin-skin-test (TST) is of limited diagnostic value (Newton et al., 2008; Haimi-Cohen et al., 2001). The high quality reference standard molecular system, Xpert MTB/RIF, has been recommended by the World Health Organization (WHO) for TB diagnosis. However, the method requires expert handling of expensive laboratory equipments, which is impractical in less-developed nations. Moreover, the quality of evidence is low for Xpert MTB/RIF based diagnosis of EPTB in children (WHO, 2014). Thus, simple, inexpensive and rapid molecular methods that require minimal instrumentation and training are required for accurate diagnosis of childhood EPTB cases in resource-limited settings.

Toward this end, loop-mediated isothermal amplification (LAMP) assay has emerged as an attractive technique and has been

* Corresponding author at: Department of Molecular Medicine, National Institute of Tuberculosis and Respiratory Diseases, Sri Aurobindo Marg, New Delhi 110 030, India.

E-mail address: drparaslr@gmail.com (P. Singh).

<https://doi.org/10.1016/j.mimet.2019.01.016>

Received 30 October 2018; Received in revised form 27 January 2019; Accepted 28 January 2019

Available online 28 January 2019

0167-7012/ © 2019 Elsevier B.V. All rights reserved.

Table 1
Sequence of primers and PCR conditions for amplification of Mtb genes by conventional PCR.

Gene	Primer sequence (5' to 3')	PCR product size (bp)	PCR conditions
IS6110	F: CCTGCGAGCGTAGGGCTCGG; R: CTCGTCCAGCGCCGCTTCGG	123	94 °C for 5 min.; 40 cycles of 94 °C for 1 min., 65 °C for 1 min., 72 °C for 1 min.; 72 °C for 10 min.
Pab	F: ACCACCGAGCGGTTCCGCTGA; R: GATCTGCGGGTCGTCCCAGGT	419	94 °C for 5 min.; 40 cycles of 94 °C for 1 min., 65 °C for 1 min., 72 °C for 1 min.; 72 °C for 10 min.
MPB64	F: TCCGCTGCCAGTCGTCTTCC; R: GTCTCGCGAGTCTAGGCCA	240	94 °C for 5 min.; 40 cycles of 94 °C for 1 min., 65 °C for 1 min. 30 s., 72 °C for 1 min. 30 s.; 72 °C for 10 min.

recommended by the WHO for diagnosis of tuberculosis (WHO, 2016). The LAMP assay takes about an hour to give results, which can be visualized with naked eye. However, evidence remains limited for EPTB cases (WHO, 2016), which are paucibacillary in nature and often remain undetected by high quality gold standard techniques and culture methods. There have been only few studies that aimed at analyzing use of LAMP assay in EPTB cases and that too in pediatric population. Thus, in our present study, we intended to analyze and compare diagnostic accuracy of LAMP assay with the other molecular and microbiological methods in clinically suspected EPTB pediatric patients visiting the Out Patient Department (OPD) of our tertiary care TB hospital in Delhi.

2. Materials and methods

2.1. Study design and sample collection

The study was conducted at the National Institute of Tuberculosis and Respiratory Diseases (NITRD), New Delhi, India following approval by the institutional ethics committee. The patients visiting the Out Patient Department of NITRD, and suspected of extra-pulmonary tuberculosis were investigated. A total of 154 children, of which 86 were boys and 68 were girls during 2015–2017 were enrolled for the present study as per the below-mentioned criteria. *Inclusion criteria:* Clinically suspected EPTB cases of either gender aged 18 years or below. *Exclusion criteria:* Clinically unsuspected EPTB cases of either gender aged above 18 years.

2.2. Sample processing and microbiological confirmation of TB

The samples collected from the OPD of NITRD, New Delhi were immediately transferred to the laboratory of Department of Molecular Medicine, NITRD for further processing. The sample processing varied as per the sample type. Sterile specimens, such as cerebrospinal fluid, peritoneal fluid, pleural fluid, or lymph node, were simply re-suspended (after homogenization wherever required) in saline and concentrated by centrifugation; while the non-sterile samples, such as, urine, or pus were decontaminated by treatment with NALC-NaOH as per the standard protocol (Kent and Kubica, 1985) described below.

2.2.1. NALC-NaOH decontamination

A mixture containing equal volumes of 4% sodium hydroxide and 2.94% sodium citrate was prepared followed by addition of 0.5% (for a 0.25% final concentration) of *N*-acetyl-L-cysteine. Equal volume of this mixture was added to each sample and allowed to stand at room temperature for 15 min. Precaution was taken not to exceed the incubation time for > 15 min., so as to avoid any unintended killing of *Mycobacterium* spp. About 40–45 ml of phosphate buffer (pH 6.8) was added to the tube and swirled for proper mixing. The mixture was centrifuged at 4500 RPM for 15 min. The supernatant was discarded and the pellet was used for further testing.

2.2.2. Microbiological confirmation of TB

Smear of all the specimens of the EPTB site were subjected to Ziehl-Neelsen staining (ZNS) followed by microscopy and culture.

2.3. DNA extraction

DNA from the decontaminated specimens was isolated using the Qiagen's QIAamp DNA mini kit (QIAGEN, Hilden, Germany) as per the manufacturer's instructions. Briefly, the pellet was transferred to a 1.5 ml microcentrifuge tube followed by addition of 180 µl of ATL buffer and 20 µl of proteinase K (10 mg/ml). Post incubation at 56 °C/260 RPM for either overnight or 2–3 h, 200 µl of AL buffer was added to the mixture and vortexed for 15 s. After incubation at 70 °C for 10 min., the tube was briefly spun followed by addition of chilled 100% ethanol. The tube was pulse-vortexed and transferred to a spin-column (provided along with the kit) and centrifuged at 8000 RPM at 4 °C for 1 min. The filtrate was discarded followed by washings with 500 µl each of AW1 and AW2 buffers. Finally, the DNA was eluted in 50 µl of TE buffer in fresh 1.5 ml microcentrifuge tubes by centrifugation at 8000 RPM/4 °C for 1 min. in two rounds and labeled as Elute 1 and Elute 2 respectively. The concentration of extracted DNA was estimated by spectrophotometric analysis (IMPLEN NanoPhotometer P-300) and accordingly working dilutions of DNA were prepared and stored at –20 °C until further use.

2.4. Molecular diagnosis

2.4.1. Conventional PCR for genes: IS6110, Pab, MPB64

The isolated bacterial DNA was subjected to conventional polymerase chain reactions for detection of each of the 3 Mtb genes, viz., IS6110, Pab and MPB64. The reaction mixture contained 50–100 ng of sample DNA, 2.5 mM of PCR buffer, 200–400 µM dNTP mix, 50–100 ng of each primer (forward & reverse), 1 U of *Taq* polymerase in a 25 µl of final volume. The reaction conditions and details of primers for each gene are listed in the Table 1.

2.4.2. Nested PCR

Nested PCR was performed using the commercially available 3B BIOTUB Kit (3B BlackBio Biotech India Ltd., India) as per the manufacturer's protocol. Briefly, the 'First PCR' reaction was set-up using 4.5 µl DNA samples as template along with 2× Multiplex Master mix (15 µl), 5× MTB PM-1 (6 µl) and 8-MOP solution (4.5 µl) in a final volume of 30 micro liters. The reaction tubes were placed in a pre-heated (94 °C) thermal cycler and the amplification was performed at 94 °C for 15 min, 30 cycles of 94 °C/20 s, 60 °C/20 s & 72 °C/30 s, with final extension at 72 °C for 5 min.

The 'Second PCR' reaction (nested PCR) mix consisted of 2× Multiplex Master mix (15 µl), 4× MTB PM-2 (7.5 µl), 8-MOP solution (6 µl) and 1.5 µl of the 'First PCR' product in a final volume of 30 µl. The nested PCR mix was transferred to a pre-heated (94 °C) thermal cycler and the amplification was done with the following conditions: 94 °C/15 min, 35 cycles of 94 °C/20 s, 62 °C/30 s & 72 °C/30 s, and a final extension at 72 °C for 5 min. Both positive and negative controls (provided with the kit) were included for each round of PCR. The PCR products were fractionated in 2% of low EEO agarose gel (Pronadisa, Laboratories Conda S.A., Madrid, Spain) for analysis of the target gene amplification.

2.4.3. LAMP assay

Commercially available Nu-LAMP TB kit (RAS Lifesciences Pvt. Ltd., Hyderabad, India) was used to perform LAMP assay as per the manufacturer's protocol. The kit is intended to detect the strains of *Mycobacterium tuberculosis* complex (*Mycobacterium africanum*, *Mycobacterium bovis*, *Mycobacterium canettii*, *Mycobacterium microti*, *Mycobacterium tuberculosis*) in body fluids. The loop mediated isothermal amplification was performed on PCR machine/heating block and the end point detection was done under UV light (254 nm).

The LAMP assay reaction mixture was set-up as per the manufacturer's instructions followed by incubation at 65 °C for 60 min. Positive and negative controls (provided along with the kit) were included in each run of the assay. Apart from these, two additional negative controls, viz., non-tubercle mycobacteria and DNA from healthy control (HC), were also included in each run. The individuals with no signs, symptoms or history of mycobacterial infection were considered healthy controls for the test. The results were interpreted by naked human eye under visible spectrum or UV radiation.

2.5. Statistical analysis

Comparison of TB positivity rate was done by direct counting method. Parameters for diagnostic accuracy were determined using MedCalc, version 18.9.1 (MedCalc software bvba, Ostend, Belgium). Efficiency of LAMP assay was compared to Composite Reference Standard, which included culture, IS6110-PCR and MPB64-PCR, among different specimen types using Chi-square test. A p-value $\leq .05$ was considered statistically significant for all the analyses.

3. Results and discussion

3.1. Study population and clinical specimens

A total of 154 subjects participated in the study, of which 55.8% (n = 86) were males and 44.1% (n = 68) were females with mean age 13 ± 5 years for both the genders. The site of infection ranged from neck, spine, to the abdomen. The major type of sample was pleural fluid in males and lymph node in female patients (Fig. 1).

3.2. Molecular vs. microbiological TB diagnosis

Of the total 154 clinically suspected EPTB cases, only 5% or 11% could be confirmed microbiologically as TB-positive by smear microscopy or culture respectively. On the contrary, at least 46.7% specimens were confirmed TB-positives by molecular diagnostic tests. Thus, a 4-fold difference was observed in diagnostic positivity rate between molecular and microbiological methods.

Of the 154 specimens, 85.7% (n = 132) samples were smear negative and culture negative; and of these 132 specimens, at least 58 (44%) came out to be positive by molecular tests (Table 2). The overall

maximum positivity rate for TB by molecular tests was 70%; nearly 6-fold higher than that by microbiological tests. Hence, a marked difference could be observed in the sensitivity of molecular diagnosis as compared to either smear microscopy or bacterial culture in childhood EPTB cases. This becomes especially relevant in context to those cases that come out negative by smear and culture and remain undiagnosed and untreated, thus constituting “hidden” TB burden in the population. Such cases can be efficiently diagnosed by the LAMP assay, which requires minimal use of equipment. The LAMP assay results did not differ under visible light and UV light (Fig. 2), hence direct visualization with naked eye under visible light provides a rapid read out of the results and reduces the overall turn-around-time. Further, with all the controls being well-diagnosed, the LAMP assay may be considered as an accurate technique for identification of “hidden” TB cases.

Among the smear- and culture-positive & smear positive culture negative group, LAMP assay demonstrated 100% positivity for TB. This is in concordance with a previous study wherein sensitivity of *M. tuberculosis* LAMP in smear-positive and culture-positive sputum samples was 97.7% (Boehme et al., 2007). Such results reflect that LAMP is a beneficial technique for clinical confirmation in cases positive by culture and also in those where the bacterial load may be low. In the smear- and culture- negative EPTB specimens, nested PCR demonstrated the highest positivity rate (66%) followed by IS6110-PCR (62%). The LAMP assay exhibited a fold lower positivity at 58%, which may be regarded as fairly acceptable considering that not all specimens may be true TB-positives under the group. Results from the other 2 groups (Table 2) showed exact efficiency of all the molecular tests except Pab-PCR. This is in accordance with the earlier reports describing Pab gene as an inefficient PCR target for diagnosis of TB (Sharma et al., 2013 and Singh et al., 2014) in comparison to IS6110 or MPB64 genes.

LAMP assay results were at par with the IS6110-, MPB64- & nested PCRs with 100% sensitivity in smear positive cases. These results are in concordance with previous studies (Ou et al., 2014; Habeenzu et al., 2017; Joon et al., 2015) that demonstrate LAMP assay as a faster, cheaper and fairly efficient technique for diagnosis of tuberculosis. Ou and colleagues demonstrated that LAMP assay could detect Mtb in spot sputum specimens with 70.67% sensitivity (Ou et al., 2014) in PTB patients in China. In a separate study, LAMP assay was assessed to have 82.3% sensitivity for diagnosis of PTB in sputum samples of Moroccan TB suspects (Bentaleb et al., 2016). As compared to smear microscopy, comparable sensitivity and specificity of LAMP assay (96.9% & 96.5%) than the Xpert MTB/RIF (95.4% & 93.9%) was reported (Habeenzu et al., 2017).

Since there is lack of any gold-standard technique for diagnosis of EPTB in children, we analyzed the diagnostic efficiency of LAMP test in reference to a composite reference standard (CRS) which comprised of culture, IS6110-PCR and MPB64-PCR. Samples were graded according to ‘Any Positive’ rule, wherein, the samples positive by any of the 3 tests were regarded as TB positive, while the samples that were negative by all the 3 tests were considered TB negative. Upon comparison with CRS

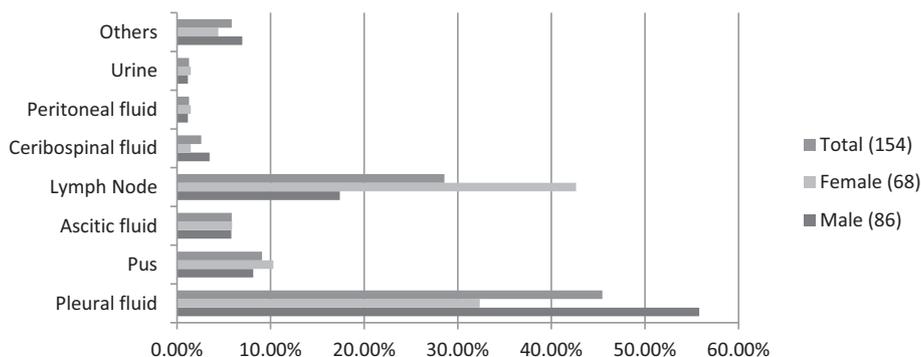


Fig. 1. Types of EPTB specimens analyzed in the study.

Table 2
Category-wise distribution of specimens positive for TB by different molecular assays.

	Smear +ve culture +ve (n = 3)	Smear +ve culture -ve (n = 5)	Smear -ve culture +ve (n = 14)	Smear -ve culture -ve (n = 132)	Total cases (n = 154)
IS6110-PCR +ve specimens	3 (100)	5 (100)	12 (85.7)	83 (62.8)	103 (66.8)
MPB64-PCR +ve specimens	3 (100)	5 (100)	13 (92.8)	81 (61.3)	102 (66.2)
Pab-PCR +ve specimens	3 (100)	2 (40)	9 (64.2)	58 (43.9)	72 (46.7)
Nested PCR +ve specimens	3 (100)	5 (100)	13 (92.8)	87 (65.9)	108 (70.1)
LAMP assay +ve specimens	3 (100)	5 (100)	14 (100)	77 (58.3)	99 (64.2)

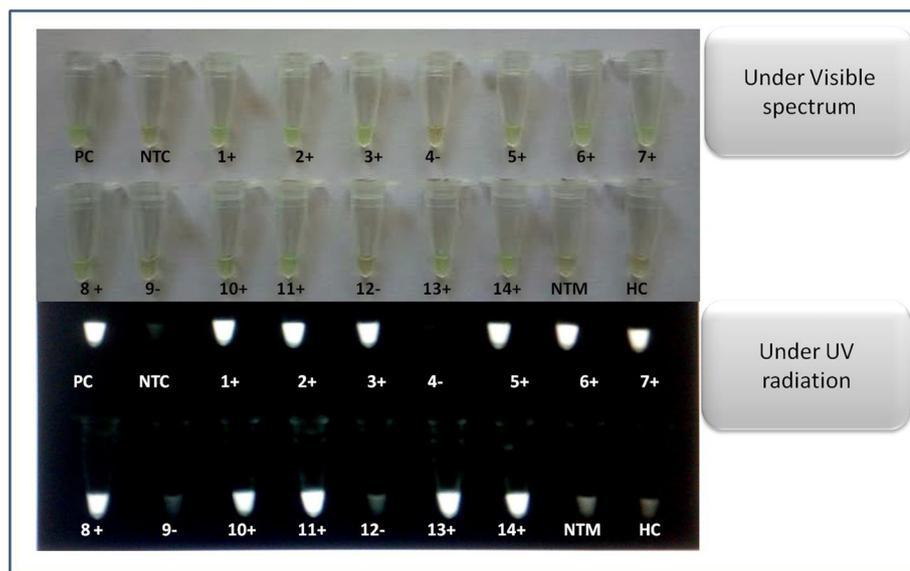


Fig. 2. End-point results of LAMP assay for detection of Mtb DNA in clinical specimens. The LAMP assay results were visualized under both visible and Ultra-violet (UV) spectrum. PC is positive control; NTC is no-template control; NTM is non-tubercle *Mycobacteria*; and HC is Healthy control. The numerical against each tube indicates sample number and the '+' or '-' signs indicate test positive or test negative respectively. Under short wave UV radiation, the TB-positive samples or PC showed fluorescent green color while no color was observed in NTC, NTM or HC.

(Table 3) or culture alone (data not shown), we found that the Nu LAMP TB assay demonstrated highly significant ($p < .001$ and $p = .003$) sensitivity (80% and 100% respectively) and specificity (78% and 40.1% respectively) in our study. This is in contrast to the findings reported by Ghosh and colleagues (Ghosh et al., 2017), who found the Nu LAMP TB kit to be 95.6% sensitive and 95.4% specific in EPTB specimens as compared to the culture based diagnosis. This non-concordance may be attributed to the sample type-based variation and subject population. The major (55.5%) specimen type in the study by Ghosh et al. was the skin punched biopsy and the study population possibly comprised both adults and children as no data was given on the age of the study subjects. In contrast, the major specimen type in our study was pleural fluid and the study was conducted on childhood cases only (≤ 18 years). In 2 separate reports conducted on frozen CSF specimens from Indian TB patients, 88% and 92% sensitivity and 80% and 61% specificity was recorded in comparison to CRS (Nagdev et al., 2011) and culture alone (Modi et al., 2016) respectively. In another study, LAMP assay was reported to be 93.3% sensitive and 91.9% specific for diagnosis of EPTB in different clinical specimens, such as, urine, blood, lymph node, etc. (Joon et al., 2015). The research group further improved their in-house LAMP assay that demonstrated a higher

sensitivity (94.4%) and specificity (97.2%) in PTB cases, however, the EPTB specimens showed comparatively lower sensitivity (86.67%) and specificity (94.04%) (Joon et al., 2017). In Chinese population, LAMP assay demonstrated 43.02% sensitivity comparable to real time fluorescent quantitative PCR for diagnosis of tubercular meningitis (Sun et al., 2017). In a multi-targeted LAMP assay, 90% sensitivity and 100% specificity was reported for diagnosis of tubercular lymphadenitis in Indian population (Sharma et al., 2016). Overall, the type of EPTB specimen (fresh/frozen and EP site), target population and assay method (i.e. target gene) seem to account for the variable sensitivity and specificity of the LAMP assay. All of these studies suggest that LAMP assay can be an excellent tool for rapid diagnosis of extra-pulmonary tuberculosis.

With reference to the CRS, nested PCR showed higher sensitivity (85%) but lower specificity (71%) than the LAMP assay (Table 3). This is in accordance with the fact that nested PCR is a valuable technique for specimens with low bacterial load, i.e. upto 1 MTB copy of genome (Aryan et al., 2010).

Further, we compared the LAMP assay based diagnosis with that of the GenXpert in 43 childhood specimens and 32 adult specimens (as we could generate GenXpert data only for these 75 specimens). The LAMP

Table 3
Comparison of diagnostic accuracy of LAMP assay with composite reference standard.

Test	CRS + ve (n)	CRS -ve (n)	Sensitivity (%)	Specificity (%)	χ^2 - value	p-value
LAMP assay +ve	90	9	79.65	78.05	41.14	< 0.001
LAMP assay -ve	23	32				
95% CI			71.04–86.64	62.39–89.44		
Nested PCR +ve	96	12	84.96	70.73	41.91	< 0.001
Nested PCR -ve	17	29				
95% CI			77.01–90.9	54.46–83.87		

Abbreviation: CRS- composite reference standard; χ^2 - values are Yates corrected.

Table 4
Diagnostic efficiency of LAMP test vs GenXpert (total n = 75).

Test	GenXpert + ve (n)	GenXpert – ve (n)	Sensitivity (%)	Specificity (%)	χ^2 - value	p-value
Adults (n = 32)						
LAMP assay + ve	13	10	86.7	41.2	1.83	0.176
LAMP assay – ve	2	7				
95% CI			66.8–97.5	23.7–50.8		
Children (n = 43)						
LAMP assay + ve	9	17	81.8	46.9	1.74	0.186
LAMP assay –ve	2	15				
95% CI			52–96.7	36.6–52		
Total (n = 75)						
LAMP assay + ve	22	27	84.6	44.9	5.29	0.021
LAMP assay – ve	4	22				
95% CI			68.1–94.7	36.1–50.3		

χ^2 - values are Yates corrected. Bold Signifies statistically significant p-values < 0.5.

assay demonstrated 82% and 87% sensitivity and 47% and 41% specificity in childhood and adult cases respectively (Table 4). The overall sensitivity of LAMP assay was 84.6% with 45% specificity in EPTB cases in comparison to the GenXpert. Although GenXpert is a highly sophisticated technique, it has been well-documented that the paucibacillary EPTB specimens do not present as ideal samples for the same (Eddabra and Ait Benhassou, 2018). The sensitivity of GenXpert is low in smear-negative PTB specimens and special populations, like, HIV-positives, children and extra-pulmonary TB. Hence, the comparison of LAMP assay with GenXpert (which tends to miss true positive EPTB cases) may not be very reliable. Moreover, mechanical errors have been reported during sample processing for Xpert MTB/RIF (Habeenzu et al., 2017; Nhu et al., 2013), and thus make this method unsuitable for peripheral laboratories.

Of the 43 childhood cases, only 11 (25.5%) cases were positive by GenXpert, of which 8 were rifampicin sensitive, 2 rifampicin resistant; and for 1 sample drug susceptibility was indeterminate. Of the 8 RIF sensitive cases, 7 were positive by LAMP assay. Also 1 RIF resistant and 1 indeterminate childhood case were positive by LAMP assay.

Further analysis was done to evaluate if the specimen type plays any role in performance of the Nu LAMP TB assay (Table 5). For this comparison, results of LAMP assay were compared to the CRS based diagnosis using Chi-square test. Among the pleural fluid samples, statistically significant difference was observed between LAMP assay and CRS with 80% sensitivity and 85% specificity. The highest positivity rate of the LAMP assay was in pus specimens at 92.8% followed by ‘other’ sample types, such as, blood, gastric aspirate, etc. and lymph node. In pleural fluid and ‘other’ specimens, LAMP assay showed statistically significant ($p \leq .05$) sensitivity (80% and 100% respectively) and specificity (85% and 100% respectively). The lymph node specimens also exhibited fairly significant ($p = .057$) sensitivity (74.2%) and specificity (61.5%). Perhaps, the low sample numbers in other different specimen categories led to statistically insignificant results.

3.3. Choosing the right assay

The ‘right assay’ refers to a rapid, easy to perform, cheaper technique with reasonable sensitivity and specificity. Smear microscopy is a tedious and less efficient process that is prone to human errors during scanning of the slides. Furthermore, the technique becomes less sensitive in specimens where the bacterial load is < 10,000 bacilli/ml of the test sample, and this is always the case in extra-pulmonary tuberculosis specimens. However, due to limited resources rapid diagnosis of MTB in high TB-burden developing countries relies on smear microscopy (Ghosh et al., 2017). Culture methods are reliable as these can detect lower bacterial load (10–100 bacilli/ml of concentrated specimen) than smear microscopy (Gupta et al., 2016; Davies and Pai, 2008) and aids in generation of data on conventional drug susceptibility test and

identification of *Mycobacterium* species. However, the culture methods are not 100% sensitive owing to the possible loss of bacterial load while decontamination of EPTB samples. The newer rapid culture methods, like liquid culture system enhance the yield by 10% over solid culture method; however, the associated high cost and need for safe disposal of the radioactive waste hinders its use in peripheral laboratories (Purohit and Mustafa, 2015). Thus, simpler, cost-effective, safe, rapid and efficient diagnostic methods are the need-of-the-hour to unveil the “hidden” TB cases in high-TB burden and resource-poor.

Evolution of molecular methods has revolutionized the diagnosis of tuberculosis (Yu et al., 2018); however, much improvement needs to be done to obtain the required fool-proof TB diagnosis (Nagai et al., 2016). It is well-known that nested PCR is more sensitive and specific than the conventional PCR method. The sensitivity of the nested PCR kit that was used in this study was < 10 copies/reaction and no cross-reactivity was observed with common human pathogenic bacteria other than *Mycobacterium tuberculosis*. In our study, the nested PCR exhibited higher sensitivity (85%) to the LAMP assay. However, the nested PCR involves setting-up of two PCR reactions, which lengthens the turn-around-time for diagnosis. The conventional single-gene PCRs (IS6110- & MPB64-PCRs) also take at least 120 min run-time.

In contrast, LAMP assay requires much lesser time, approx. 60 min. (half the time of single-gene PCR) for a single run in a basic aluminum heat block; thereby reducing the overall time for generation of diagnostic results. Moreover, the results can be visualized with naked eyes under visible light (Fig. 2), without the requirement of additional equipment. In our present study, LAMP assay exhibited 80% sensitivity and 78% specificity. LAMP assay showed an overall 64% positivity rate with analytical sensitivity of 5 copies per reaction and specificity to the strains of *Mycobacterium tuberculosis* complex. Furthermore, efficient detection of *M. tb* in different types of EPTB specimens displayed LAMP assay as an apparent preference for TB diagnosis. Other than being much easier and less time-consuming than nested PCR, the LAMP assay is simpler, easy to conduct, has lesser turn-around-time, requires only basic equipment for single temperature incubation, and demonstrates comparable sensitivity and specificity.

4. Conclusions

Molecular diagnosis of tuberculosis is imperative along with routine smear microscopy and culture methods. Our findings from the present work provide further evidence that LAMP assay is a promising technique for EPTB diagnosis in childhood cases. And that the technique can be implemented in developing countries where TB burden is high and resources and infra-structure is scarce.

Table 5
Comparison of diagnostic efficiency of LAMP assay on different types of EPTB specimens.

Type of specimen	N	Smear microscopy ^a +ve	LJ culture +ve	IS6110-PCR +ve	MPB64-PCR +ve	Nested PCR +ve	LAMP assay +ve	CRS +ve	χ^2 value ^b	p-value [@]	Sensitivity	Specificity
Pleural fluid	70	5 (7.1)	6 (8.5)	44 (62.8)	46 (65.7)	46 (65.7)	43 (61.4)	50 (71.4)	22.8	< 0.001	80%	85%
Pus	14	0	4 (28.5)	13 (92.8)	12 (85.7)	13 (92.8)	13 (92.8)	13 (92.8)	0	1	92.3%	n/a
Ascitic fluid	9	0	0	6 (66.6)	5 (55.5)	4 (44.4)	4 (44.4)	7 (77.7)	0.39	0.53	57.10%	100%
Lymph Node	44	2 (4.5)	1 (2.2)	31 (70.4)	29 (65.9)	35 (79.5)	28 (63.6)	31 (70.4)	3.627	0.057	74.20%	61.5%
Cerebrospinal fluid	4	0	1 (25)	2 (50)	4 (100)	1 (25)	3 (75)	4 (100)	0	1	75%	n/a
Peritoneal fluid	2	0	0	1 (50)	0	1 (50)	1 (50)	1 (50)	0	1	100%	100%
Urine	2	0	0	0	0	0	0	0	0	1	n/a	100%
Others	9	1 (11.1)	5 (55.5)	6 (66.6)	6 (66.6)	8 (88.8)	7 (77.7)	7 (77.7)	4.14	0.042	100%	100%

The values in parenthesis represent percentages; ^aSmear microscopy was performed on EPTB site smear sample; N = Total number of samples tested in the study; ^b χ^2 test: for comparison between LAMP assay and CRS for TB diagnosis; the χ^2 value is Yates corrected; [@]p-value < .05 was considered significant. Bold Signifies statistically significant p-values < 0.5.

Funding information

The authors thank Dr. Rohit Sarin, Director, National Institute of Tuberculosis and Respiratory Diseases, New Delhi, India for providing necessary funds and materials for conducting this research work.

Conflicts of interest

The authors declare that they have no competing interests for publication of this manuscript.

References

Ahmed, T., Sobhan, F., Ahmed, A.M.S., 2008. Childhood tuberculosis: a review of epidemiology diagnosis and management. *Infect. Dis. J. Pakistan* 17, 52–60.

Aryan, E., Makvandi, M., Farajzadeh, A., Huygen, K., Bifani, P., Mousavi, S.L., et al., 2010. A novel and more sensitive loop-mediated isothermal amplification assay targeting IS6110 for detection of Mycobacterium tuberculosis complex. *Microbiol. Res.* 165 (3), 211–220.

Bentaleb, E.M., Abid, M., El Messaoudi, M.D., Lakssir, B., Ressami, E.M., Amzazi, S., 2016. Development and evaluation of an in-house single step loop-mediated isothermal amplification (SS-LAMP) assay for the detection of Mycobacterium tuberculosis complex in sputum samples from Moroccan patients. *BMC Infect. Dis.* 16 (1), 517.

Boehme, C.C., Nabeta, P., Henostroza, G., et al., 2007. Operational feasibility of using loop-mediated isothermal amplification for diagnosis of pulmonary tuberculosis in microscopy centers of developing countries. *J. Clin. Microbiol.* 45, 1936–1940.

Davies, P.D., Pai, M., 2008. The diagnosis and misdiagnosis of tuberculosis. *Int. J. Tuberc. Lung Dis.* 12 (11), 1226–1234.

Eddabra, R., Ait Benhassou, H., 2018. Rapid molecular assays for detection of tuberculosis. *Pneumonia (Nathan)* 10, 4.

Ghosh, P.K., Chakraborty, B., Maiti, P.K., Ray, R., 2017. Comparative evaluation of loop-mediated isothermal amplification and conventional methods to diagnose extra-pulmonary tuberculosis. *Ann. Trop. Med. Public Health* 10, 160–164.

Gupta, S., Goyal, R., Bareja, R., Behara, R.N., 2016. Evaluation of various culture and staining techniques for the detection of extra pulmonary tuberculosis. *Int. J. Res. Med. Sci.* 4 (9), 3982–3987. (Sep). [10.18203/2320-6012.ijrms20162919](https://doi.org/10.18203/2320-6012.ijrms20162919).

Habenzu, C., Nakajima, C., Solo, E., Bwalya, P., Kajino, K., Miller, M., Kurosawa, Y., Mudenda, V., Kasonka, L., Suzuki, Y., Matsuba, T., 2017. Evaluation of in-house loop-mediated isothermal amplification for tuberculosis diagnosis compared with Xpert MTB/RIF. *J. Infect. Dev. Ctries.* 11, 440–444. <https://doi.org/10.3855/jidc.7730>.

Haimi-Cohen, Y., Zeharia, A., Mimouni, M., Soukhman, M., Amir, J., 2001. Skin indurations in response to tuberculin testing in patients with non-tuberculous mycobacterial lymphadenitis. *Clin. Infect. Dis.* 33 (10), 1786–1788.

Joon, D., Nimesh, M., Saluja, D., 2015. Loop-mediated isothermal amplification as alternative to PCR for the diagnosis of extrapulmonary tuberculosis. *Int. J. Tuberc. Lung Dis.* 19, 986–991.

Joon, D., Nimesh, M., Varma-Basil, M., Saluja, D., 2017. Evaluation of improved IS6110 LAMP assay for diagnosis of pulmonary and extra pulmonary tuberculosis. *J. Microbiol. Methods* 139, 87–91.

Kent, P.T., Kubica, G.P., 1985. *Public Health Mycobacteriology: A Guide for the Level III Laboratory*. U.S. Department of Health and Human Services, Washington, DC.

Modi, M., Sharma, M., Sharma, A., Sharma, N., Sharma, S., et al., 2016. Multitargeted loop-mediated isothermal amplification for rapid diagnosis of tuberculous meningitis. *Int. J. Tuberc. Lung Dis.* 20, 625–630.

Nagai, K., Horita, N., Yamamoto, M., Tsukahara, T., Hideyuki, N., Tashiro, K., et al., 2016. Diagnostic test accuracy of loop-mediated isothermal amplification assay for Mycobacterium tuberculosis: systematic review and meta-analysis. *Sci. Rep.* 6, 39090.

Nagdev, K.J., Kashyap, R.S., Parida, M.M., Kapgate, R.C., Purohit, H.J., Taori, G.M., et al., 2011. Loop-mediated isothermal amplification for rapid and reliable diagnosis of tuberculous meningitis. *J. Clin. Microbiol.* 49, 1861–1865.

Nelson, L.J., Wells, C.D., 2004. Global epidemiology of childhood tuberculosis. *Int. J. Tuberc. Lung Dis.* 8 (5), 636–647.

Newton, S.M., Brent, A.J., Anderson, S., Whittaker, E., Kampmann, B., 2008. Paediatric tuberculosis. *Lancet Infect. Dis.* 8 (8), 498–510.

Nhu, N.T., Ha, D.T., Anh, N.D., Thu, D.D., Duong, T.N., Quang, N.D., Lan, N.T., Quyet, T.V., Tuyen, N.T., Ha, V.T., Giang, D.C., Dung, N.H., Wolbers, M., Farrar, J., Caws, M., 2013. Evaluation of Xpert MTB/RIF and MODS assay for the diagnosis of paediatric tuberculosis. *BMC Infect. Dis.* 13, 31.

Ou, X., Li, Q., Xia, H., Pang, Y., Wang, S., et al., 2014. Diagnostic accuracy of the PURE-LAMP test for pulmonary tuberculosis at the county-level laboratory in China. *PLoS One* 9 (5), e94544. <https://doi.org/10.1371/journal.pone.0094544>.

Purohit, M., Mustafa, T., 2015. Laboratory diagnosis of extra-pulmonary tuberculosis (EPTB) in resource-constrained setting: state of the art, challenges and the need. *J. Clin. Diagn. Res.* 9 (4), EE01–EE06.

Sharma, K., Gupta, V., Bansal, R., Sharma, A., Sharma, M., Gupta, A., 2013. Novel multi-targeted polymerase chain reaction for diagnosis of presumed tubercular uveitis. *J. Ophthalmic Inflamm. Infect.* 3, 25–31.

Sharma, M., Sharma, K., Sharma, A., Gupta, N., Rajwanshi, A., 2016 Sep. Loop-mediated isothermal amplification (LAMP) assay for speedy diagnosis of tubercular lymphadenitis: the multi-targeted 60-minute approach. *Tuberculosis (Edinb)* 100, 114–117.

Singh, P., Singh, M., Tayal, D., Myneedu, V.P., Bhalla, M., Adlakha, P., Sarin, R., 2014.

- The clinical utility of polymerase chain reaction and adenosine deaminase (ADA), for the diagnosis of pleural tuberculosis: Indian scenario. *Immunol. Infect. Dis.* 2 (2), 13–21.
- Sun, W.W., Sun, Q., Yan, L.P., Zhang, Q., 2017. The application of IS6110-based loop-mediated isothermal amplification (LAMP) in the early diagnosis of tuberculous meningitis. *Oncotarget* 8, 57537 ± 42.
- Togun, T., Kampmann, B., Pai, M., 2017. Diagnosis of childhood tuberculosis. In: Reference Module in Biomedical Sciences, <https://doi.org/10.1016/B978-0-12-801238-3.64157-0>.
- WHO (World Health Organization), 2014. Guidance for National Tuberculosis Programmes on the Management of Tuberculosis in Children, 2nd edition. Available on. http://www.who.int/tb/publications/childtb_guidelines/en/, Accessed date: 3 October 2018.
- WHO (World Health Organization), 2016. The Use of Loop-Mediated Isothermal Amplification (TB-LAMP) for the Diagnosis of Pulmonary Tuberculosis. Policy Guidance. Available on. <http://www.who.int/tb/publications/lamp-diagnosis-molecular/en/>, Accessed date: 3 October 2018.
- WHO (World Health Organization), 2017. ANNEX 2: Country Profiles For 30 High TB Burden Countries. Available on. http://www.who.int/tb/publications/global_report/gtbr2017_annex2.pdf?ua=1.
- WHO (World Health Organization), 2018. Tuberculosis. Key Facts. Updated September 18, 2018, Available on. <http://www.who.int/news-room/fact-sheets/detail/tuberculosis>, Accessed date: 29 September 2018.
- Yu, G., Shen, Y., Zhong, F., Ye, B., Yang, J., Chen, G., 2018. Diagnostic accuracy of the loop-mediated isothermal amplification assay for extrapulmonary tuberculosis: a meta-analysis. *PLoS One* 13 (6), e0199290. <https://doi.org/10.1371/journal.pone.0199290>.