

Evaluation of Loop Mediated Isothermal Amplification (Lamp) Assay for Diagnosis of Mycobacterium Tuberculosis in Sputum Samples

*Dr. SM Tushar Alom¹, Dr. Kanak Jyoti Mondal², Dr. Shirin Tarafder³,
Dr. Farah Anjum Sonia⁴, Dr. Md Shoriful Islam⁵, Dr. Abu Naser Ibne Sattar⁶

1. Medical Officer, Department of Transfusion Medicine, Khulna Medical College Hospital, Khulna

2. Registrar, Department of Medicine, Khulna Medical College Hospital, Khulna

3. Associate Professor, Department of Microbiology, Bangabandhu Sheikh Mujib Medical University, Dhaka

4. Medical Officer, Department of Transfusion Medicine, Bangabandhu Sheikh Mujib Medical University, Dhaka

5. Resident, Department of Nephrology, Dhaka Medical College Hospital, Dhaka

6. Associate Professor Department of Microbiology, Bangabandhu Sheikh Mujib Medical University, Dhaka

Corresponding Author: S M Tushar Alom

Abstract: The lack of rapid and accurate diagnostic testing is an important impediment to global tuberculosis control. For rapid diagnosis of pulmonary tuberculosis, majority of the laboratories are restricted to AFB sputum smear microscopy but the low sensitivity is the major problem. Recently, loop mediated isothermal amplification (LAMP) assay has been developed as a novel technique for nucleic acid amplification. LAMP can amplify DNA with high specificity and efficiency under isothermal conditions using six sets of primers that recognize eight distinct regions on the target sequence.

All samples were done for AFB sputum smear microscopy, culture in Lowenstein- Jensen media, conventional PCR targeting IS6110 and LAMP assay targeting 16SrRNA.

Among the 114 clinically suspected pulmonary tuberculosis cases, LAMP assay showed the highest rate of positive results. LAMP assay were positive in 90(78.95%) cases, conventional PCR were positive in 72(63.16%) cases, culture was positive in 58(50.87%) cases and AFB smear were positive in 56(49.12%) cases. Out of 114 clinically suspected pulmonary tuberculosis cases, all smear positive, culture positive and conventional PCR positive cases were LAMP assay positive. Among 114 clinically suspected pulmonary tuberculosis cases, 17 cases were negative by all methods but were positive by LAMP assay. Considering culture as a gold standard the sensitivity and specificity of LAMP assay were 100% and 42.85% respectively.

Due to its easy operation without sophisticated equipment, this assay may facilitate the identification of M. tuberculosis for the confirmation of diagnosis in clinically suspected but smear negative as well as culture negative pulmonary tuberculosis cases.

Key Words: Loop Mediated Isothermal Amplification Assay(LAMP), Pulmonary Tuberculosis, 16srRNA, IS6110, Mycobacterium tuberculosis.

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I. Introduction

Tuberculosis (TB) remains a major public health problem worldwide and is a leading cause of death in developing countries. The burden of tuberculosis rests almost entirely on the developing world; 55% of all incident cases were reported in Asia and 30% in Africa. (WHO, 2009) Approximately 9 million new cases and 1.4 million TB deaths were estimated globally in 2011. (WHO, global tuberculosis report, 2013). Early diagnosis of *Mycobacterium tuberculosis* (MTB) in sputum is important not only for initiating treatment but also for curbing the disease transmission to others in the community (Brodie and Schluger, 2005). Sputum smear microscopy, although widely available in primary health care settings in developing countries like Bangladesh, is the least expensive, simple and relatively easy to perform but has both the problems of sensitivity and specificity (Tiwari et al., 2007). Smear microscopy only detects about 50% of cases (WHO, 2009). Culture, though the gold standard for diagnosis, takes 3–6 weeks for growth to occur in Lowenstein-jensen (LJ) media and 1–6 weeks in automated systems like BACTEC and MGIT 960 (Sethi et al. 2013) thus lead to unacceptable delaying of diagnosis. Among the various newly introduced method of amplification, PCR is highly sensitive assay which may even detect 1-10 organisms within 3-6 hours in different clinical specimens (Verma et al. 1995) but it

requires sophisticated laboratory settings, skilled personnel, expensive, time consuming and have risk of post PCR contamination. Recently, loop mediated isothermal amplification (LAMP) assay has been developed as a novel technique for nucleic acid amplification (Notomi et al., 2000). LAMP can amplify DNA with high specificity and efficiency under isothermal conditions using six sets of primers that recognize eight distinct regions on the target sequence. Unlike PCR, LAMP reaction does not require a denatured DNA template and relies on auto cycling strand displacement DNA synthesis by a *Bst* DNA polymerase (Nagamine et al., 2001). It can be performed using a simple water bath, thus eliminating the need for a thermo cycler. The large amount of DNA generated in less than an hour and positive LAMP reaction can be visualized with the naked eye, without need for gel electrophoresis (Mori et al., 2001). Thus, LAMP is a simple, rapid, cost effective highly sensitive and specific DNA amplification technique for early diagnosis of TB which does not require special instruments, modern laboratory facilities and can be done in areas where these facilities are not available (Kohan *et.al.* 2011). Bangladesh is a high burden country for Tuberculosis, and many of the TB positive cases are undiagnosed due to lack of availability of molecular methods in peripheral settings. LAMP assay is anticipated to become the dominant and most widely used manual molecular method of choice for rapid detection of *Mycobacterium Tuberculosis* in clinical sample, especially in resource poor settings where only electric supply is available. Based on this concern the aim of this study was to evaluate the efficacy of MTB specific LAMP assay targeting 16srRNA which confirm an accurate and affordable diagnostic test for Tuberculosis.

II. Material and Methods

A total of 144 sputum specimens were collected from patients having symptoms of TB attending the Tuberculosis control and training institute, Chankharpool, Dhaka as case and 30 Non-TB patients as control suffering from respiratory diseases other than tuberculosis. The cases were divided into following groups: (A) smear positive and culture positive (S+C+) ($n = 48$); (B) smear positive but culture negative (S+C-) ($n = 8$); (C) smear negative but culture positive (S-C+) ($n = 10$); (D) smear negative and culture negative, which were clinically and radio-logically confirmed (S-C-) ($n=48$). All samples were processed with N-Acetyl-L-Cysteine Sodium Hydroxide(NALC-NaOH) method and then stained with Z-N stain and cultured in L-J media. DNA were extracted from homogenized sputum concentrate using a DNA extraction kit (GenolyseVer 1.0 Hainlifescience GmbH/Hard wiesenstr1/72147, Germany) according to manufacturer instruction. PCR was also performed to amplify 123 bp sequences unique to *M. tuberculosis* insertion sequence (IS6110) as described (Narayanan et al.2001) The primer pair used was MTB-F 5'-CCT GCG AGC GTA GGC GTC GG 3' and MTB-R 5' CTC GTC CAG CGC CGC TTC GG -3'. LAMP assay in the present study targeted 16S rRNA gene of MTB, using one set of each inner primers, outer primers, and loop primers. . The primers were used: Forward Outer Primer (F3)-CTGGCTCAGGACGAACG,Backward Outer Primer (B3)—GCTCAT CCCACACCGC, Forward Inner Primer (FIP)—CACCCACGTGTTACTCATGCAAGTCGAACGGA AAGGTCT, Backward Inner Primer (BIP)—TCGGGATAAGCCTGGACCACAAGACATGCATC CCGT, Forward Loop Primer(FLP)—GTTCGCCACTCGAGTATCTCCG,Backward Loop Primer (BLP)—GAAACTGGGTCTAATACCGG. Loopamp reaction tubes containing dried DNA amplification reagents were labeled properly. (Ready MTB-LAMP kit from EIKEN CHEMICAL CO, LTD JAPAN). (Operated on ice) Dispensed 15 μ l of primer mix to each reaction tube. Added 10 μ l of extracted DNA in each reaction tube ,After closing the cap, the reaction tubes were inverted to transfer the solution to the cap, and allowed it to stand on ice for 2 minutes. kept it in heat block at 65°C for 40 minutes. Then Kept it at 95°C for 2 minutes for Enzyme deactivation. After 40 minutes' incubation at 65°C the LAMP reaction tubes were placed on the UV Lamp (Wavelength at 240 nm). Observed the reaction tube with the eyes protected by eyeglass. Positive control (PC HIP) and negative control were used in each lot which was supplied with commercial kit. Samples were considered as LAMP assay Positive when the LAMP product produced green fluorescence within the reaction tubes indicates MTB DNA amplification. Samples were considered as LAMP assay negative when the LAMP product produced no fluorescence within the reaction tubes indicate no MTB DNA amplification. All data were analyzed by using computer based SPSS (Statistical Package of Social Science) software version: 20 with a 95% confidence interval (95% CI). The results of individual tests were compared by Chi-square test. P value < 0.05 was taken as minimum level of significance. This study was ethically approved by the Institutional Review Board, of Bangabandhu Sheikh Mujib Medical University (BSMMU).

III. Results

Out of 114 clinically suspected pulmonary tuberculosis cases (Group A-D), LAMP assay were positive in 90 (78.95%) cases, Conventional PCR were positive in 72 (63.16%) cases, Culture were positive in 58(50.87%) cases and Smear were positive in 56(49.12%). Thus LAMP detecting the maximum number of cases among these assays. All 30 control patients were negative by all methods like Smear, Culture, Conventional PCR and LAMP assay. Both Smear positive and Culture positive 48 cases were enrolled in Group-A, where 48(100%) cases were positive by Conventional PCR and LAMP assay. Smear positive but

culture negative 8 cases were enrolled in Group B. In this group out of 8, 7(87.5%) cases were positive by PCR and 8(100%) cases were positive by LAMP assay. 10 cases were enrolled in Group-C (Smear negative but Culture positive). Out of these 10 cases, 10(100%) cases were positive by PCR and 10(100%) cases were positive by LAMP assay. In Group-D, both Smear and Culture negative cases were 48. In this group, 7(14.5%) cases were PCR positive and 24(50%) cases were LAMP assay positive.

Correlation among results of smear and culture with LAMP assay in clinically suspected pulmonary tuberculosis cases were shown in Table -IV. Out of 56 AFB smear positive cases, 56(100%) were positive by LAMP assay and out of 58 AFB smear negative cases, 34(58.6%) cases were LAMP assay positive and 24(41.4%) cases were LAMP assay negative. Of 58 culture positive cases, 58(100%) cases were LAMP assay positive and in 56 culture negative cases, 32(57.14%) cases were LAMP assay positive and 24(42.86) cases were LAMP assay negative. Correlation between conventional PCR test results and LAMP assay among clinically suspected pulmonary tuberculosis cases are illustrated in Table-V. Among 72 PCR positive samples, LAMP assay were positive in 72(100%) cases. Out of 42 PCR negative cases, LAMP assay were positive in 18(42.85%) cases, and 24(57.15%) cases were negative by LAMP assay. Performance characteristics of smear, PCR and LAMP Assay were done considering Culture as a gold standard. It was seen that out of 58 culture positive cases, 48(82.7%) were true positive and 10(17%) were false negative by AFB smear; 58(100%) cases were true positive and 0(0%) were false negative by PCR; 58(100%) cases were true positive and 0(0%) cases were false negative by LAMP Assay.

Out of 56 culture negative cases, 8(14.28%) cases were false positive and 48(85.71%) cases were true negative by AFB smear; 14(25%) cases were false positive and 42(75%) cases were true negative by PCR; 32(57.14%) cases were false positive and 24(42.8%) cases were true negative by LAMP Assay. The sensitivity and specificity of AFB smear were 82.76% and 85.71% respectively. The sensitivity and specificity of PCR were 100% and 75% respectively and the sensitivity and specificity of LAMP Assay were 100% and 42.85% respectively (Table VI).

IV. Discussion

The present study was designed to see the efficacy of LAMP assay for early, rapid and accurate diagnosis of mycobacterium tuberculosis DNA in sputum samples in comparison to AFB smear, culture and conventional PCR.

In this study the overall sensitivity and specificity of AFB smear were 82.76% and 85.71% respectively. The sensitivity and specificity of PCR were 100% and 75% respectively and the sensitivity and specificity of LAMP assay were 100% and 42.85% respectively.

A study was conducted in Nepal which showed that the sensitivity of Mycobacterium tuberculosis detection in LAMP assay in C+ specimens was 100%. This result suggests that MTB-LAMP may be superior to the acid-fast smear test and comparable with bacterial culture testing, though the number of culture positive and smear negative samples was not large enough to strongly conclude this. MTB-LAMP specificity in culture negative samples was 94.2%. These results from culture negative patients may suggest that MTB-LAMP is possibly highly sensitive in the detection of a small number of tubercle bacilli released from hidden foci in sputum. *M. tuberculosis* was detected by MTB-LAMP in all culture positive and a number of culture negative samples. Compared with results from standard culture testing, acid-fast staining and chest radiography, this study results demonstrate the high clinical performance of MTB-LAMP in the rapid detection of *M. tuberculosis* from sputum samples (Pandey *et al.*2008). In above mentioned study the specificity of LAMP assay was higher than the present study because their study subjects were smear positive, culture positive and radiologically diagnosed pulmonary tuberculosis cases.

A multi-center study on *M. tuberculosis* detection showed the feasibility of using the LAMP method in developing countries. The study evaluated LAMP assay targeting the gyrB gene and using a simplified manual DNA extraction method. The sensitivity of MTB-LAMP was 97.7% in S+ and C+ sputum samples and 48.8% in S- and C+ sputum samples (Boehme *et al.*, 2007). The findings of the above study are lower than the present study where the sensitivity is 100% in C+ and S+ samples and 100% for S- and C+ sputum samples.

A study was performed in Sri Lanka which showed that the sensitivity of LAMP assay was 92% and specificity was only 7%. This indicated a high percentage of false positives by LAMP assay as compared to culture (Senarath *et al.*2014).

Another study was performed in Bangalore, India where the LAMP assay showed excellent sensitivity (96.7%) and specificity (100.0%) for those samples with analogous culture and smear results. LAMP showed poor sensitivity (22.2%) and specificity (33.3%) for the samples with discordant culture and smear results. The overall sensitivity (79.5%) and specificity (93.8%) of LAMP was slightly lower than that of the smear microscopy (82.1% and 96.9% respectively) when compared to culture. LAMP showed a sensitivity and specificity of 96.9% and 100.0% respectively for smear positive samples. The specificity of LAMP for smear negative samples was 93.6%. The sensitivity of LAMP for smear negatives could not be calculated due to the

limited number of samples. Both the LAMP and smear microscopy specificity were 79.5% and 100% respectively (Geojith *et al.* 2010).

A study was conducted by Sethi *et al.* in 2013 which evaluated a MTB-specific in-house LAMP assay targeting the 16SrRNA of MTB complex (MTBC), and compared it with IS6110 PCR and conventional techniques in suspected cases of pulmonary TB. The sensitivity of LAMP was found to be 98.4% in S+C+ (smear positive, culture positive) samples, which is in agreement with a multi centric study conducted by Boehme *et al.* in 2007, on MTB detection, which showed the feasibility of using LAMP in developing countries. The study evaluated a prototype LAMP targeting the *gyrB* gene and found the sensitivity of LAMP to be 97.7% in S+C+ samples, which is comparable to these results. However, the sensitivity was 48.8% in S-C+ sputum samples, which was lower than that found in the present study (100%). Similarly, a study carried out in Nepal by Pandey *et al.* in 2008 which has shown sensitivity of LAMP to be 96.1% in S+C+ and 85% in S-C+ samples. According to the study by Aryan *et al.* 2009, LAMP can be used to provide confirmation of clinically suspected pulmonary TB cases where smear is negative.

Another study was performed in Iran in 2011 which showed the sensitivity and specificity of LAMP assay in comparison with culture was 100% and 95.9% respectively (Kohan *et al.* 2011).

But in this study the specificity of LAMP assay is lower (42.85%) than the above mentioned studies because this study was done only on clinically suspected pulmonary tuberculosis cases. Moreover, the performance characteristics of LAMP assay was done considering culture as a gold standard where culture positive cases were only 58(50.87%). So the specificity of the LAMP assay becomes lower in comparison to the studies in which the LAMP assay was done on culture positive cases. The specificity of LAMP assay could be higher if Nucleic acid amplification (NAA) test can be considered as gold standard.

Yet culture remains the gold standard for laboratory confirmation of Tuberculosis, In January 22, 2009 The US Centers for Disease Control and Prevention (CDC) have issued updated guidelines for the use of nucleic acid amplification (NAA) tests in the diagnosis of tuberculosis (TB). "Since then, NAA testing has become a routine procedure in many settings because NAA tests can reliably detect *Mycobacterium tuberculosis* bacteria in specimens 1 or more weeks earlier than culture. Earlier laboratory confirmation of TB can lead to earlier treatment initiation, improved patient outcomes, increased opportunities to interrupt transmission, and more effective public health interventions. (CDC Updates Guidelines for Nucleic Acid Amplification Tests to Diagnose Tuberculosis, 2009). If the NAA test become established as the confirmatory test for diagnosis of *Mycobacterium tuberculosis* then the specificity of LAMP assay would be higher and superior among other NAA test like PCR, Gene Xpert as very low amount of DNA (5 fg μ l⁻¹ of DNA= 1 copy of DNA) can be detected by LAMP assay within 30 to 40 minutes. But in PCR amount of DNA detection limit is more (100 fg μ l⁻¹ of DNA= 20 copies of DNA) (Kohan *et al.* 2011). Gene Xpert can detect 25fg μ l⁻¹ of DNA (5 copies of *M. tuberculosis* DNA) (USAID TB CARE-II). Time required for conventional PCR is 3 to 4 hours and for Gene Xpert is at least 2 hours.

LAMP assay can be used not only for earlier clinical confirmation in culture positive cases, but also to confirm the disease in those cases where there is low bacterial load, and hence smear positivity is low (Sethi *et al.* 2013). An improved LAMP alone or together with smear microscopy can offer same day diagnosis and has the potential to reduce the drop-out rate substantially (George *et al.* 2011).

The LAMP method has now been developed as commercial kits and some of them have been adopted as the officially recommended methods for the routine identification and surveillance of pathogen in Japan (Mori *et al.* 2009). According to the WHO expert group meeting in 2013, LAMP assay for TB that could be implemented in place of microscopy to improve the accuracy of TB detection at microscopy centres and similar laboratories (WHO, Expert Group Meeting Report, Geneva: May 2013).

In conclusion, to initiate the treatment of clinically suspected pulmonary tuberculosis cases where smear and culture are negative, clinician may decide to give the empirical therapy on the basis of results of MTB-LAMP assay correlating with other supportive investigations and clinical features. So, earlier laboratory confirmation of pulmonary tuberculosis can lead to earlier treatment initiation, improved patient outcomes, increased opportunities to interrupt transmission, and more effective public health interventions.

TB-LAMP assay is a new assay which offers a manual molecular approach for the detection of *Mycobacterium tuberculosis* which seems to be feasible in peripheral laboratories following extensive training. Further research is needed to improve the specificity of LAMP assay especially for high burden TB countries. Evaluation of the TB-LAMP assay by more investigators is encouraged to enable further independent assessment.

Table –I: Group-wise comparison of conventional PCR and LAMP assay in clinically suspected pulmonary tuberculosis cases (n=114)

Group(n)	Conventional PCR n (%)	LAMP assay Positive n (%)
Group-A S+C+(48)	48(100)	48(100)
Group-B S+C-(8)	7(87.5)	8(100)
Group-C S-C+(10)	10(100)	10(100)
Group-D S-C-(48)	7(14.5)	24(50)

Note-1: S+ Smear positive,S- smear negative, C+ Culture positive, C- Culture negative.

Note-2: Total percentage exceeded the positive cases due to positivity showed among the group.

Table-II: Performance characteristics of Smear, PCR and LAMP assay compared with Culture as a gold standard (n=114)

Culture	Smear		PCR		LAMP	
	Pos No. (%)	Neg No. (%)	Pos No.(%)	Neg No.(%)	Pos No.(%)	Neg No.(%)
Positive (n=58)	48(82.7)	10(17)	58(100)	0(0)	58(100)	0(0)
Negative(n=56)	8(14.28)	48(85.71)	14(25)	42(75)	32(57.14)	24(42.8)
Sensitivity	82.76%		100%		100%	
Specificity	85.71%		75%		42.85%	

Note: Pos-positive Neg- Negative

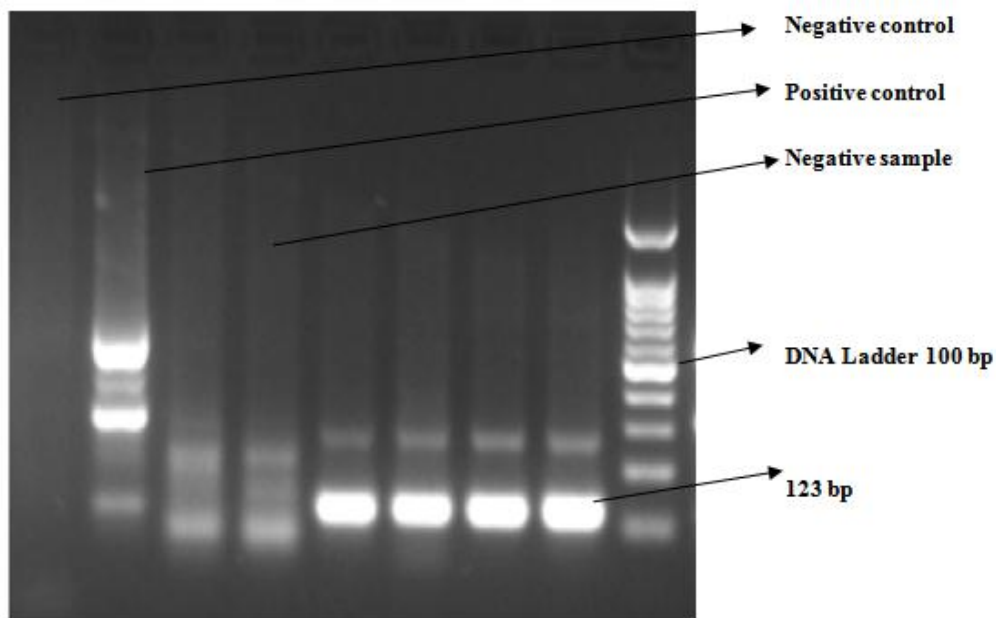


Figure:1. Amplification of 123 bp product(IS6110) of *M. tuberculosis* on 2% agarose gel by PCR.

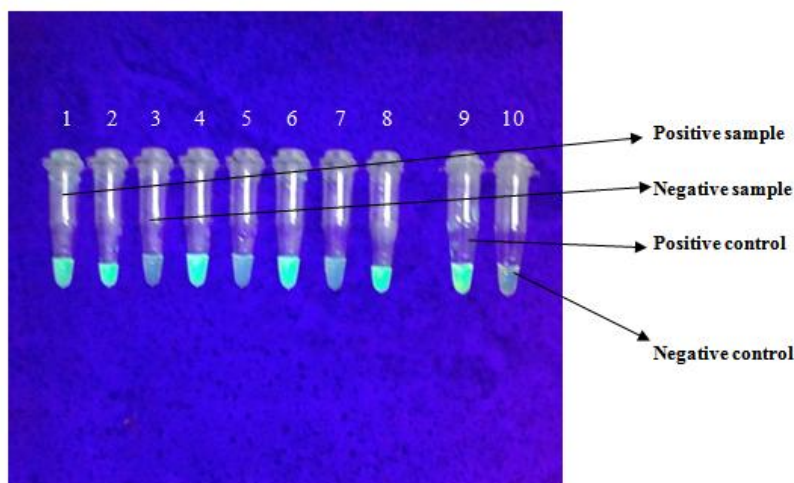


Figure: 2. Visual detection of LAMP product under UV light. From left to right tubes 1,2,4,6,8 are positive, tubes 3,5,7 are negative, tubes 9- Positive control, tubes 10- Negative control.

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