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Full Length Research Article

Diagnostic accuracy study of loop mediated isothermal amplification in the detection of *Mycobacterium tuberculosis* in Pakistan

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The study has been conducted in Armed Forces Institute of Pathology, Rawalpindi Pakistan from 2015 to 2017. The study was done to determine the adequacy of Loop-mediated Isothermal Amplification for the detection of *Mycobacterium tuberculosis*. A cross sectional study was done on 72 clinical sputum samples taken from the patient suspected of tuberculosis using a novel technique LAMP targeting IS6110 gene sequence for the first time in Pakistan. Six primers recognizing eight distinct regions on the target sequence were employed. The assay was performed in total volume of 25 µl containing primers, DNA polymerase, Fluorescent dye and sample incubated at 65°C for 60 minutes along with positive and negative controls. LAMP amplicons were detected by their fluorescence under UV light and compared with gold standard MGIT culture system and smear microscopy. Out of 72 total samples 60(83.3%) were LAMP positive and 61(84.7%) were MGIT positive. Its specificity, sensitivity is 100% and 98.36% respectively with PPV 100% and NPV 91.6%. According to our study the LAMP assay is suggested to be a potential nucleic acid based diagnostic method for TB detection in developing countries.

Keywords: Loop Mediated Isothermal Amplification (LAMP), IS6110 gene, *Mycobacterium tuberculosis*. Mycobacterium Growth Indicator Tube (MGIT)

INTRODUCTION

Tuberculosis (TB) caused by *Mycobacterium tuberculosis*, is a highly contagious disease and a major public health issue. Each year more than 8.6 million new cases and 1.3 million consequent deaths due to TB have been reported; mainly affecting population in developing countries (Organization, 2013). TB has been rampant for decades and Pakistan is ranked 6th amongst the TB high-burden countries in the world (Khan et al., 2016). The incidence of sputum smear positive TB cases is 80/100,000 per year and for other sample types it is estimated 177/100,000; which constitutes in Pakistan 5.1 percent of the cumulative national disease burden (Vermund et al., 2009). Besides efficient therapeutic regimes, rapid, accurate, and low-cost laboratory diagnostic methods are urgently needed to control TB and to reach the misdiagnosed cases (Bi et al., 2012; Organization, 2013).

There are many methods, at present, for diagnosing TB including Ziehl-Neelsen staining (ZN), conventional culturing on Lowenstein–Jensen (LJ) medium, *Mycobacterium* growth indicator tube (MGIT) culture, PCR, TB gold and T spot (Ryu, 2015); these are either less specific and sensitive, time consuming, expensive or

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need a more skilled staff, respectively. There is a need to overcome these identifiable barriers in TB diagnosis; thus, helping in containing tuberculosis in developing countries. Smear microscopy test has been done for many years for screening as well as for the diagnosis purpose. It is less expensive and the results are readily available. However, this method is less specific and sensitive as it requires 10,000 bacilli per ml and usually identifies MTB in highly infectious patients and leaves the health care workers to biosafety risk (Desikan, 2013). The test also requires more skilled staff. MGIT culture system, the novel culture method, is highly specific, sensitive and it gives results in 10 days and approached as a preferred method over conventional culturing on LJ medium (Watterson and Drobniewski, 2000). However, MGIT system needs expensive equipment and requires more skilled expertise, and complicated to operate in low cost settings. Therefore, this research work was conducted to assess the role of a novel technique, Loop-Mediated Isothermal Amplification (LAMP), which has been demonstrated as quick, easy and economical diagnostic method to detect TB earlier (Bi, et al., 2012). The method is based on the principal of accurate detection of MTB nucleic acid using 6 set of primers. It involves loop mediated amplification of MTB target DNA. It is a highly specific procedure that depends on dual DNA amplification and strand displacement activities of the Bst polymerase and a unique amplification design. The LAMP technology allows the generation of large amounts of specific amplicons at constant temperature 65 ^{o}C, leading to a high sensitivity and specificity in a simple setting. The study on this novel methodology has already been conducted earlier in countries like Japan, Thailand, Iran and India (Geojith et al., 2011; Pandey et al., 2008) but no study has been conducted in Pakistan. The study is conducted to assess the methodology, running cost, accuracy of LAMP assay to detect TB, as well as its comparison with gold standard MGIT. The method can be applied in small peripheral hospitals, basic health units and in clinical pathological laboratories in a high burden TB country like ours, thus facilitating eradication of tuberculosis.

METHODOLOGY

Sample collection

Seventy two sputum samples were collected from patients clinically suspected to have pulmonary TB. All other samples and those who were already diagnosed or on treatment were not included in the study.

The informed consent was obtained from the patients and the study was approved by the ethical committee of Armed Forces Institute of Pathology (AFIP).

MGIT culture and smear microscopy

The clinical specimens were investigated by smear microscopy and culture by MGIT system and LAMP simultaneously. The BACTEC MGIT system (Becton Dickinson Biosciences, Sparks, MD), is used as gold standard, and widely employed for the consistent and fast detection of MTB and the susceptibility as a routine in reference laboratories (Ganeswrie et al., 2004). The radiometric BACTEC MGIT test requires around ten to fourteen days (10-14 days) of incubation. It uses efficiently fluorescence based technology which is quenched with O_2. When oxygen is utilized by MTB the fluorescent dye release and gives the indication of O_2 consumption precisely.

All 72 samples collected were first subjected to decontamination and digestion by Petroff's method (Palomino and Portaels, 1998). The 0.5ml of processed sample was then inoculated in the MGIT tube containing middle brook 7H9 liquid media and incubated at 37^{o}C. The results were obtained after 10 days of incubation. Among those the positive samples were confirmed by ZN staining and further identified by TB identification device (BD diagnostics)

Lamp procedure

All the samples were assessed by LAMP assay, specifically targeting IS6110 gene.

DNA Extraction

Mycobacterial DNA extraction was done by the process described by manufacturer (Light Diagnostics). 50 µl of CSS was added to 200 µl DNA extraction reagent. The final 250µl solution was then mixed by vortex mixer for 10 sec. 250 µl isopropanol was then added to the solution and was centrifuged at 16 × 1000 G for 10 minutes. Afterwards, supernatant was drained out, while taking care that the pellet in the tube should not break. 400 µl of 70% ethanol was then added, vortex and centrifuged at 16.1 × 1000 G for 10 minutes. The supernatant was poured out. The residual pellet was incubated at 55°C for 10-15 minutes. DNA was dissolved then in 50 µl 1XTris-EDTA buffer. It was again incubated at 55°C for 10 minutes to re suspend completely.

Primer Design

The precise designing of primers is a critical step in DNA amplification using the LAMP method. A set of six primers were used for meticulous detection of MTB. It included two inner primers, Forward inward and Back Inward Primer (FIP and BIP), two outer primers (F3 and B3) and two loop primers, Forward Loop and Backward

Loop primers (FLP, BLP) recognizing six distinct regions on the target sequence. The gene that targeted was insertion sequence (IS6110) gene. The FIP primer included F2 and F1c which was complementary sequence of F1. Similarly, BIP primer contained B2 and B1c (a complementary sequence of B1). The primer sequences employed in this study are as follows (Kohan et al., 2011):

F3, 5′-AGACCTCACCTATGTGTCGA-3′;
B3, 5′-TCGCTGAACCGGATCGA-3′;
FIP, 5′-ATGGAGGTGGCCATCGTGGAAGCCTACGTGGCCTTTGTCAC-3′;
BIP, 5′-AAGCCATCTGGACCCGCCAACCCCTATCGTATGGTGGAT-3′;
FLP, 5′-AGGATCCTGCGAGCGTAG-3′; and
BLP, 5′- AAGAAGGCGTACTCGACCTG-3′.

LAMP Reaction

LAMP was carried out in a 25 µl solution total. It contained each of 0.2 µM F3 and B3, 1.6µM FIP and BIP, 0.8 µM FLP and BLP, the buffers 20mM Tris-HCl (pH: 8.8), KCl, 10mM (NH4)2SO4, 9mM MgSO4, 1.4mM dNTP, 0.8M Betaine (Sigma-Aldrich), 8U DNA polymerase (New England Biolabs, USA) and DNA sample (Kohan, et al., 2011).Then the LAMP specific primers master mix stock solution equivalent to 200 µl was prepared which contained 40 µl FIP, BIP each, 10 µl F3,B3 each, and 20 µl LF,LB each, taken from stock solution and added in 60 µl Molecular biological grade water . Afterwards, in a 1.5 ml PCR tube 10µl primers master mix was poured and 1.5µl Betaine, 0.5 µl dNTPs, 0.5µl Tris-HCl (pH: 8.8), KCl, $(NH_4)_2SO_4$, $MgSO_4$ each was added. To this solution 1.5µl *Bst* DNA polymerase, 1.5 µl Syber green fluorescent dye and 5 µl DNA extracted sample was added and kept in heat block. The reactions were carried out at 65°C and incubated in heat block for one hour. With each badge, a positive (known *Mycobacterium tuberculosis*) and negative control was included, and all safety measures to avert cross-contamination were observed.

After the incubation for 60 minutes, the amplification of DNA in the suspected MTB clinical sputum specimens (CSS) through LAMP Method was observed and interpreted. The amplified DNA in the reaction tube was identified through the visual change that was done with the naked eye and also under UV light of short wavelength due to the use of SYBR green I in reaction mixture, which turned green only if there was amplified DNA in the solution (LAMP positive); whereas absence of green color means LAMP negative. The interpretation was done along with use of positive and negative control samples. In order to further confirm the amplification, the DNA concentration in the reaction tubes was then quantified by using 'Qubit Flourometer' (Invitrogen). A positive sample in bright green color in tube labeled as + is shown in Figure 1.

RESULTS

Figure 1. Visualization of LAMP amplicons under ultraviolet light with positive and negative control on the left and the test samples on the right.
Tube labeled as "+" is Positive control, "-"is Negative control, and 1-6, are the samples run.

The LAMP method was employed on both smear positive and negative cases; and was evaluated against the gold standard MGIT test. The results from LAMP assay were rightly consistent with the results from MGIT culture. All of the smear positive and MGIT culture positive samples was also LAMP positive. Moreover, a few smear negative but culture positive was also all LAMP positive. The forty smear positive samples were MGIT culture positive as well as LAMP positive. However, 20 smear negative samples were positive on both MGIT culture and LAMP test. These results clearly showed the concordance of the LAMP test with the gold standard test. Table 1 and Figure 2 shows the comparative results and their graphical representations, respectively, of a few samples between the three procedures (smear microscopy, MGIT culture and LAMP test) employed in this study.

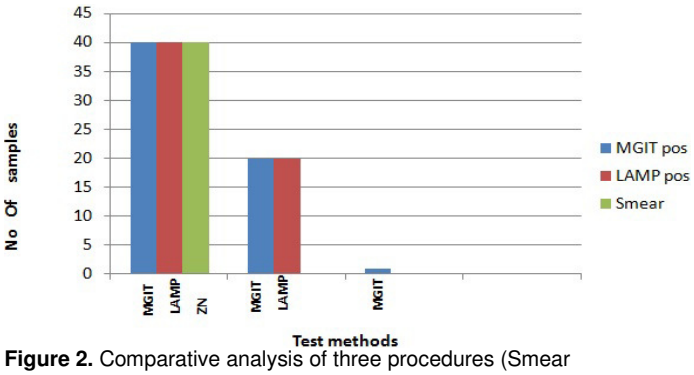

Figure 2. Comparative analysis of three procedures (Smear
Figure 2. Comparative analysis of three procedures (Smear Microscopy, MGIT Culture and LAMP Test) employed in this study. X-axis represents the three test methods and Y-axis shows he number of samples. First three bars show that 40 out of total tested samples were simultaneously smear positive, MGIT culture positive and LAMP test positive. The second two bars represent those 20 samples which are Smear negative but positive for both MGIT culture and LAMP assay. Third one represents the sample which was negative on smear microscopy and LAMP test but positive for MGIT culture.

Table 1. Comparison of Positive and Negative Results

No. of Specimens	Smear Microscopy	MGIT	LAMP
40	Positive	Positive	Positive
20	Negative	Positive	Positive
01	Negative	Positive	Negative
11	Negative	Negative	Negative

Table 2. Comparison of various experimental studies based on LAMP Method

Research Works	Specimen Numbers	Target Gene	Specificity %	Sensitivity %
Iwamoto *et al.* (2003)	66	GyrB	-	-
Boehme *et al.* (2007)	725	GyrB	99	88.2
Pandey *et al.* (2008)	200	16S rRNA	94.2	100
Zhu *et al.* (2009)(Zhu et al., 2009)	30	Rim	-	-
Aryan *et al.* (2010)	10	IS6110	-	-
Kohan *et al.* (2011)	133	IS6110	95.9	100
Present study	72	IS6110	100	98.36

Among 72 of the patients who were clinically suspected MTB 83.33% (60 out of 72) were LAMP positives, 84.7% were MGIT culture positive and 55.5% were smear positives. And as was mentioned above, among 60 positives samples, 40 were culture as well as LAMP positives while there were also 33.33% (20 out of 60) smear negatives which, however, were tested as positives on LAMP. Thus, LAMP assay average specificity was calculated 91.7% and sensitivity 100% in diagnosing MTB. The Positive and Negative predictive value (PPV and NPV, respectively) of the LAMP is 100 and 91.6% respectively. The sensitive and specificity calculated, according to true positive and true negative values, is 98.36 and 100 % respectively.

DISCUSSION

The challenge to contain rapidly prevailing tuberculosis in developing country like ours requires efficient, rapid and reliable diagnostic methods. The already employed methods including MGIT, culture and smear microscopy require expertise and specialized equipments. This will pose a hindrance in early detection of the disease. We have attempted for the first time in Pakistan to evaluate LAMP assay in diagnosing MTB in clinical specimen samples, and compare it with smear microscopy and culture system. Many researchers around the globe already have worked upon the LAMP assay in order to detect MTB among pulmonary samples and we have found out that our work is in good agreement with their findings (Table 2)

Previously, few studies were conducted on Isothermal Amplification assay by targeting gene gyrB (Boehme et al., 2007; Iwamoto et al., 2003) and 16S rRNA (Pandey et al., 2008) to detect and diagnose MTB infections. On the other hand, Aryan *et al.* (Aryan et al., 2010) studied isothermal amplification by targeting IS6110.This assay was performed on 15 clinical specimens. The detection limit was found to be 200 copies per reaction when the test was run with non-denatured DNA but it reduced to 1 copy (equal to 5fg/ µl) when the DNA utilized was heat denatured. Thus, it showed better detection limit with denatured sample. Therefore, we tested the assay with denatured DNA because higher sensitivity was achieved which was equivalent to one copy with heat-denatured template. Although the assay had lower analytical sensitivity with non-denatured DNA but still it was able to find out 5 copies of DNA. In PCR 20 copies of DNA that is equal to 100fg/µl used to be detected (Aryan et al., 2010). So, it is concluded that Isothermal Amplification has greater sensitivity of detection as well.

In our study, we found that sensitivity of isothermal amplification assay, in detecting MTB, for Culture positive specimens was 98.36% and specificity 100%. This observation demonstrates that the advanced and innovative LAMP Method, targeting the IS6110 gene, which is more sensitive than the previous study done by Boehme *et al.,* in 2007 (Boehme et al., 2007) in which they targeted gene gyrB and sensitivity of assay was found to be 88.2% (95% CI, 83.9 to 92.5). Likewise, in 2008 another study by Pandey *et al.,* (Pandey et al., 2008) with assay targeting the 16S rRNA gene, the sensitivity of LAMP was 100% in culture positive sputum samples and specificity was 94.2% in culture negative sputum samples. Furthermore, in the research work done by Kohan *et al.* (2011) (Kohan et al., 2011), the specificity of LAMP, while targeting IS6110 gene, was 95.9% that was lesser than that of Boehme *et al.* (2007) (Boehme et al., 2007) and higher than that of Pandey *et al.* (2008)

(Pandey et al., 2008) by 1.7%. But this study (Kohan et al., 2011) developed the LAMP assay showed better specificity and sensitivity in comparison with the amplification methods used in preceding studies.

Similar to the Kohan *et al.*'s (2011) (Kohan et al., 2011), this undertaken research work was done on LAMP targeting IS6110 gene in order to detect MTB among 72 CSS (clinically suspected sputum specimens). As a result, the specificity and sensitivity were worked out to be 100% and 98.36% respectively with PPV of 100% and NPV 91.6%. As far as sensitivity in our case is concerned it is slightly lower than the studies of Pandey *et al. (*2008) (Pandey et al., 2008) and Kohan *et al.* (2011) (Kohan et al., 2011). On the other hand, the specificity of the method in this study is better than that of others. However, in our case even, the improved NPV and PPV shows the accuracy of the LAMP Method.

Our study has revealed certain interesting findings: Firstly, the high sensitivity, in the present study, has certain factors to be considered. The sample tested was clinically suspected sputum specimens that have less contamination risks than other biological samples. Another important factor is the choice of primers design targeting a high-copy-number gene, i.e., *IS6110.* This is further sustained by Aryan *et al.* (2010) (Aryan et al., 2010), who showed that by designing primers for this region, 5 fg of *M. tuberculosis* DNA (equivalent to 1 copy) could be detected, performing better in comparison to the other primers used. Secondly in the current study, some of the samples (20/60) proved to be LAMP positive but smear negative, further analysis of which through the LAMP method verified that they were positive samples; and this very fact proves that LAMP is conveniently beneficial for detecting MTB even at an early stage of disease which can be neglected by the other diagnostic tests like PCR and smear microscopy.

CONCLUSION

In brief, the LAMP assay targeting IS6110 gene is convenient, practical and feasible method which is favorable for early detection of MTB in clinically suspected samples. The present study revealed, through its results, that the LAMP method has high specificity and sensitivity. And it takes only 60 minutes, as compared to 3 to 4 hours that are a usual time for the other molecular tests like PCR, while making it valuable for tertiary health care centers which require prompt results. Consequently, the test is simple, less-time consuming and does not require any specialized equipment and skilled staff, hence can be performed in routine clinical labs; especially in highly burdened countries like Pakistan, to detect MTB, which can lead to swift and effective management of the tuberculosis.

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REFERENCES

Aryan E, Makvandi M, Farajzadeh A, Huygen K, Bifani P, Mousavi SL, Romano M (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.

Bi A, Nakajima C, Fukushima Y, Tamaru A, Sugawara I, Kimura A, Suzuki Y (2012). A rapid loop-mediated isothermal amplification assay targeting hspX for the detection of Mycobacterium tuberculosis complex. Jap. J. Infect. Dis. 65(3): 247-251.

Boehme CC, Nabeta P, Henostroza G, Raqib R, Rahim Z, Gerhardt M, Hase T (2007). Operational feasibility of using loop-mediated isothermal amplification for diagnosis of pulmonary tuberculosis in microscopy centers of developing countries. J. Clin. Microbiol. 45(6): 1936-1940.

Desikan P (2013). Sputum smear microscopy in tuberculosis: Is it still relevant? The Ind. J. Med. Res 137(3): 442.

Ganeswrie R, Chui C, Balan S, Puthucheary S (2004). Comparison of BACTEC MGIT 960 system and BACTEC 460 TB system for growth and detection of Mycobacteria from clinical specimens. The Malaysian J. Pathol. 26(2): 99-103.

Geojith G, Dhanasekaran S, Chandran SP, Kenneth J (2011). Efficacy of loop mediated isothermal amplification (LAMP) assay for the laboratory identification of Mycobacterium tuberculosis isolates in a resource limited setting. J. Microbiol. Methods. 84(1): 71-73.

Iwamoto T, Sonobe T, Hayashi K (2003). Loop-mediated isothermal amplification for direct detection of Mycobacterium tuberculosis complex, M. avium, and M. intracellulare in sputum samples. J. Clin. Microbiol.41(6): 2616-2622.

Khan MB, Mashhood AA, Qureishi SM (2016). Recent advances in the management of tuberculosis. J. Pak. Assoc. Dermatol. 15(2): 151-160.

Kohan L, Shahhosseiny MH, Razavi MR, Parivar K, Moslemi E, Werngren J (2011). Evaluation of loop mediated isothermal amplification for diagnosis of Mycobacterium tuberculosis complex in clinical samples. Afr. J. Biotechnol. 10(26): 5096-5101.

Organization WH (2013). Global tuberculosis report 2013: World Health Organization.

Palomino J, Portaels F (1998). Effects of decontamination methods and culture conditions on viability of Mycobacterium ulcerans in the BACTEC system. J. Clin. Microbiol.36(2): 402-408.

Pandey BD, Poudel A, Yoda T, Tamaru A, Oda N, Fukushima Y, Sapkota B (2008). Development of an in-house loop-mediated isothermal amplification (LAMP) assay for detection of Mycobacterium tuberculosis and evaluation in sputum samples of Nepalese patients. J. Med. Microbiol. 57(4), 439-443.

Ryu YJ (2015). Diagnosis of pulmonary tuberculosis: recent advances and diagnostic algorithms. Tuberculosis and respiratory diseases. 78(2): 64-71.

Vermund SH, Altaf A, Samo RN, Khanani R, Baloch N, Qadeer E, Shah SA (2009). Tuberculosis in Pakistan: A decade of progress, a future of challenge. J. Pak. Med. Assoc. 59(4): 1-8.

Watterson S, Drobniewski F (2000). Modern laboratory diagnosis of mycobacterial infections. J. Clin. Pathol. 53(10): 727-732.

Zhu RY, Zhang KX, Zhao MQ, Liu YH, Xu YY, Ju CM, Chen JD (2009). Use of visual loop-mediated isotheral amplification of rimM sequence for rapid detection of Mycobacterium tuberculosis and Mycobacterium bovis. J. Microbiol. Methods. 78(3): 339-343.