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Evaluation of IS6110 PCR and LAMP in Sputum Samples for Diagnosis of Pulmonary Tuberculosis

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***Abstract*— Loop-Mediated Isothermal Amplification (LAMP) is an upcoming molecular method for TB diagnosis. It uses six primers to amplify DNA isothermally and results can be observed visually. The present study is focused on the comparison of performance of LAMP and PCR targetting IS6110 with conventional methods- smear microcopy and culture. Sputum samples were collected from 230 TB suspects which were analysed by all the above methods. LAMP detected the maximum number of TB cases (111/119) as compared to PCR (97/119), smear (98/119) or culture (105/119) Overall the sensitivity and specificity of LAMP was 93.2% (95% CI: 87.1-97.0%) and 97.1% (95% CI: 91.8-99.4%) respectively.These results are very encouraging and suggest that LAMP has the potential to serve as efficient method of diagnosis for TB.**

Keywords*— PCR, LAMP, IS6110, *Mycobacterium tuberculosis

I. INTRODUCTION

Mycobacterium tuberculosis (M.tb) mediated TB is the leading cause of death due to a single infectious disease worldwide [1]. TB diagnosis and treatment is a serious public health challenge for developing countries. Early and accurate diagnosis of TB can help control the transmission of the disease from infected to healthy individuals. However, limited resources and technical expertise hinder the use of advanced methods of TB diagnosis in countries like India. Hence health care workers have to fall back on conventional, time consuming and less reliable diagnostic methods like smear microscopy and culture. Smear microscopy is the most widely used technique in developing countries but lacks sensitivity (10^4bacteria/ml of sample) [2] . TB culture, the gold standard for diagnosis is sensitive (10-100 bacteria/ml of sample) but takes 3-6 weeks in the case of solid culture and 1-6 weeks with liquid culture to give positive results [3]. A solution to this problem is the advent of rapid and reliable nucleic acid amplification tests (NAAT) like PCR which can detect *M.tb* in patient specimens with high specificity and sensitivity [4]. One such NAAT is loop mediated isothermal amplification assay or LAMP which has marked advantages over PCR. Discovered by Notomi *et al.,* (2000) [5], it employs *Bst* DNA polymerase to amplify template DNA using 3 pairs of specifically designed primers. Since it is an isothermal process it can be carried out in a simple laboratory water bath or heating block as compared to PCR which requires a thermocycler. It has higher specificity as it uses 3 pairs of primers and synthesizes large amounts of DNA in less than an hour. Hence the amplified products can be detected visually using fluorescent dyes like SYBR Green 1 whereas agarose gel electrophoresis is essential to analyze PCR results. Hence, LAMP has potential to become an effective tool for TB diagnosis in developing countries. WHO recommends extensive evaluation studies before LAMP is approved for routine use [6]. This study focuses on the comparison of LAMP and PCR targeting IS6110 which is the most widely distributed DNA sequence in the *M.tb* genome (copy number upto 25 reported) [7].

II. MATERIALS AND METHODS

A. Study Design

Consecutive sputum samples were collected from 230 suspected pulmonary TB patients (complaining of cough for more than 2 weeks, fever, chest pain and breathlessness) visiting the DOTS (Directly Observed Treatment Short Course) centre of Post Graduate Institute of Medical Education Research, Chandigarh, in the period January, 2013-March, 2014. Early morning sputum sample was collected from patients in wide mouth, screw capped containers. The samples were transported to the lab on the same day (within 1-2hours) and smears were prepared followed by staining with Z-N method [8]. Thereafter, the samples were decontaminated by NALC-NaOH method [8] and used for culture inoculation on L-J medium and DNA was extracted by method described by van Embden *et al*., (1993) [9] and stored at -20ºC.

B. IS6110 PCR & LAMP

The primers described by Eisenach *et al.*, (1990) [10] (Table 1) were used for IS6110 PCR assay. The PCR mix (25µl) composition was: 1X PCR buffer, 200 pmol of each primer (Sigma, US), 100µM dNTP (Bangalore Genei, India), 1.5mM $MgCl_2$, 1U Taq DNA Polymerase (Bangalore Genei, India) and 100ng template DNA. Amplification was carried out as follows:

Initial denaturation	95°C (3 min)
Denaturation (30 cycles)	95°C (80 seconds)
Annealing	55°C (80 seconds)
Primer extension	72°C (140 seconds)
Final extension	72°C (3 min)

The presence of 123 bp amplification product was observed by electrophoresis of the reaction product on 1% EtBr stained agarose gel.

Table I: IS6110 PCR & LAMP Primers

S.No	PCR Primers	Sequence
1	FP	5'-CCTGCGAGCGTAGGCGTCGG-3'
2	RP	5'-CTCGTCCAGCGCCGCTTCGG-3'
	LAMP Primers	
3	IS-FIP	5'-ATGGAGGTGGCCATCGTGGAAG-CTACGTGGCCTTTGTCAC-3'
4	IS-BIP	5'-AAGCCATCTGGACCCGCCAA-CCCCTATCCGTATGGTGGAT-3'
5	IS-FLP	5'-AGGATCCTGCGAGCGTAG-3'
6	IS-BLP	5'-AAGAAGGCGTACTCGACCTG-3'
7	IS-FOP	5'-AGACCTCACCTATGTGTCGA-3'
8	IS-BOP	5'-TCGCTGAACCGGATCGA-3'

Similarly IS6110 LAMP was carried out with primers described previously by Aryan *et al.*, (2000) [11]. The LAMP assay composition (25µl) was: *Bst* Polymerase Thermopol buffer (1X), betaine (0.8M), IS-FIP(1.6mM) and IS-BIP (1.6mM), IS-FOP(0.2mM) and IS-BOP(0.2mM), IS-FLP(0.8mM) and IS-BLP (0.8mM), dNTPs (2mM),$MgSO_4$(6mM), 8U *Bst* DNA Polymerase (New England Biolabs, MA, US) and template DNA. The assay conditions were: 65ºC for 60 minutes andterminated by incubating the tube at 90ºC for 2minutes. The results were analysed by colour change of fluorescent dye SYBR Green I (Invitrogen Inc., CA, US) (10µl of 100 times diluted) and confirmed by electrophoresis of the reaction products in 1% agrose gel which showed a typical ladder like pattern of amplicons.

To avoid any contamination negative controls were also set up with each reaction which contained all the components excluding any DNA. Positive control contained confirmed DNA from *M.tb*H37Rv. Assay was set-up in a thermocycler (Techne R Genius Thermocycler, Cambridge, UK). The stained gel was examined under UV light (AlphaImager™3400, India) to look for the DNA bands using 100bp ladder.

C. Statistical Analysis

TB culture is considered as gold standard for diagnosis. The available methods for TB detection are not 100% sensitive. Therefore, the confirmed TB cases were defined as either smear or culture positive and clinically positive patients which responded to antitubercular treatment [12]. The calculation of sensitivity, specificity, and other performance indicators was done according to confirmed TB cases using standard formulae and online MedCalc software.

III. RESULTS

Out of the 230 patients, cultures for sputum samples from seven TB suspects were contaminated and were excluded from the study. The samples were divided into 5 groups (A) AFB positive by smear microscopy and LJ culture positive; (B) AFB positive by smear microscopy but LJ culture negative; (C) AFB negative by smear microscopy but LJ culture positive; (D) AFB negativeby smear microscopy and LJ culture negative but clinically confirmedand (E) Non-TB cases (n=104) in which microbiological, radiological and clinical evidence were negative for TB (controls). The distribution of patients in each group is as described in Table II. The age of the patients ranged from 14 years to 80 years. The male to female ratio was 1:2.5.

Table II: Grouping of patients and performance of PCR and LAMP

Group	Status	No. of patients	LAMP	PCR
A	AFB positive by smear microscopy and LJ culture positive (S+C+)	90	90	83
B	AFB positive by smear microscopy but LJ culture negative (S+C-)	8	7	4
C	AFB negative by smear microscopy but LJ culture positive (S-C+)	15	11	10
D	AFB negative by smear microscopy and LJ culture negative but clinically confirmed (S-C-)	6	3	0
E	Controls (n=104) Non-TB cases	104	3	2

From the 119 confirmed TB patients (Groups A-D) 98 were smear positive and 105 were culture positive. Besides these there were 6 clinically positive patients which were neither smear nor culture positive. IS6110 PCR correctly detected *M.tb* in 97 patients and 111 were positive by IS6110 LAMP (Figure 1). In group D (Clinically positive TB) PCR could not detect any patients and LAMP detected 3. Hence the overall sensitivity of LAMP came out to be 93.2% (95% CI: 87.1-97.0%) as compared to 81.5% (95% CI: 73.3-88.0) of PCR. The positive and negative predictive values for PCR were 97.9% (95% CI: 92.4-99.4) and 82.2% (95% CI: 76.0%-

87.1%) and LAMP were 97.3% (95% CI: 92.3-99.1%) and 92.6% (95% CI: 86.5-96.1%). The individual groupwise sensitivity of LAMP and PCR is shown in table III.

Table III: Group wise comparison of sensitivities of PCR and LAMP

Sensitivity →Groups	A	B	C	D
PCR (95% CI)	92.2% 84.6% to 96.8%	50.0% 15.7% to 84.3%	66.6% 38.3% to 88.1%	0 0.0% to 45.9%
LAMP (95% CI)	100% 95.9% to 100.0%	87.5% 84.6% to 99.6%	73.3% 44.9% to 92.2%	50.0% 11.8% to 88.1%

In 111 samples of group E or the control group, LAMP was positive for 3 samples. However, on close evaluation of clinical history no clear indication suggestive of TB was found. These three samples were considered false positive because of which specificity of the test fell below 100%. PCR was also positive for 2 samples. The specificity for both PCR and LAMP is shown in table 4. The proportion of agreement between PCR and LAMP came out to be 93.0% (95% CI 88.5- 95.9%) (Table IV).

Table IV: Comparison of IS6110 PCR and LAMP on different parameters

	Sensitivity	Specificity	PPV	NPV
LAMP (95%CI)	93.2% (87.1-97.0%)	97.1% (91.8-99.4%)	97.3% (92.3-99.1%)	92.6% (86.5-96.1%)
PCR (95%CI)	81.5 (73.3-88.0%)	98.0% (93.2-99.7%)	97.98% (92.4-99.4)	82.2% (76.0%-87.1%)

Table V. Proportion of agreement between IS6110 PCR and LAMP

	PCR Positive	**PCR Negative**	**Kappa value**
LAMP Positive	99	15	93%
LAMP Negative	0	101	

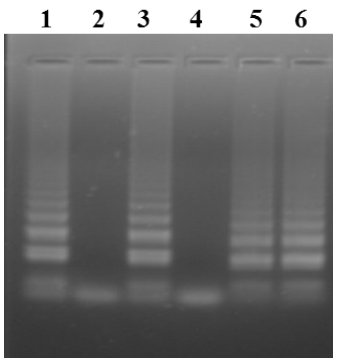

Figure 1: LAMP for representative samples. Lane 1 and 2 show positive and negative controls respectively. Lanes 3-6 show LAMP for sputum samples.

IV. DISCUSSION

The rise of TB to epidemic proportions in the 21st century has made it a major health issue worldwide [1]. Problems like the emergence of drug resistance strains on one hand and failure to contain infection in the TB endemic areas on the other are serious roadblocks in the race to defeat TB. The problem of TB in developing countries like India is multi-dimensional. Sophistication of labs and efficient technical training of the staff is not possible due to limited resources of the governments. Inspite of flourishing private sector in medical and diagnostic field, reaching out to the remote and rural areas becomes an issue. Hence, providing quality healthcare facilities to all sections of society is an uphill task [2]. The commendable efforts of government through RNTCP for controlling TB coupled with faster and economical diagnostic method together can overcome this challenge. Hence the importance of new NAATs for early detection of TB and timely initiating ATT in patients is apparent. LAMP is such a test which fulfills all these requirements. The results of IS6110 LAMP were quite encouraging.

As expected of any NAAT, LAMP and PCR detected more TB cases than any of the other conventional diagnostic methods. The sensitivity of IS6110 LAMP was 93.28% and specificity was 97.12% much in accordance with other evaluation studies from our lab ([13]-[17]). LAMP was found to be most sensitive for S+C+ samples with 100% sensitivity. Kohan *et al.,*(2011) [18] and Kaewphinit *et al*.,(2013) [19] also performed LAMP studies targeting IS6110 with similar outcomes. Both showed the sensitivity to be 100% with culture positive samples which decreased in culture negative cases. It is worth mentioning here that Kaewphinit *et al.,* (2013) used four primers for LAMP and combined it with a lateral flow dip-stick assay to achieve that sensitivity. This shows that LAMP can be used to confirm clinically suspected patients. However, the sensitivity decreased in case of smear negative sputum samples but was still better than conventional methods. The sensitivity of LAMP in S+C- samples was 87.5 and in S-C+ samples was 73.3%. The performance of LAMP

was always better than PCR in each group (50% and 66.6% in S+C- and S+C- respectively). This is because of higher DNA amplification in LAMP. In the clinically positive group (S-C-) sensitivity of LAMP was 50% as it positively detected only 3 of the 6 TB patients. In the light of ATT response reviewed after 2 months, all these patients showed a positive response to ATT. Inspite of contradicting studies [20] where sensitivity of LAMP in this group was poor, our study demonstrates that LAMP can pick up extremely small amounts of bacterial DNA in the sputum sample. The presence of multiple copies of the IS repeat element is another factor which enhances its efficiency.

The proportion of agreement between PCR and LAMP came out to be 93.0%. According to Altman (1999) [21] an agreement more than 40-60% between two tests shows a moderate strength of agreement and 61-80 represents a good strength. Hence, our study shows a very good strength of agreement between PCR and LAMP.

V. Conclusion

We have established that the performance of IS6110 LAMP far exceeds that of conventional and PCR methods. This technique has major advantages in terms of sensitivity, affordable cost and time consumption. It can become a prominent and prevalent routine test for TB diagnosis especially in developing countries. However it needs extensive evaluation in different laboratories to ensure its performance.

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