Evaluation of In-House Loop-Mediated Isothermal Amplification (LAMP) Assay for Rapid Diagnosis of *M. tuberculosis* in Pulmonary Specimens

Sunil Kumar Sethi,^{1*} Sumeet Singh,² Sunil Kumar Dhatwalia,¹ Rakesh Yadav,¹ Abhishek Mewara,¹ Malkit Singh,¹ Rupinder Tewari,² and Meera Sharma¹

¹Department of Medical Microbiology, Postgraduate Institute of Medical Education and Research, Chandigarh, India

²Department of Microbial Biotechnology, Panjab University, Chandigarh, India

Background: Loop-mediated isothermal amplification (LAMP) assay has come forward as a rapid, cost-effective molecular technique for diagnosis of tuberculosis (TB) in developing countries. This study evaluated *Mycobacterium tuberculosis*-specific in-house LAMP assay targeting 16S rRNA and compared it with other conventional tests and nucleic acid amplification assay (IS6110 PCR). **Methods:** A total of 133 sputum specimens (103 from suspected pulmonary TB cases and 30 from non-TB controls) were subjected to conventional tests, IS6110 PCR and 16S rRNA LAMP assay. **Results:** Of the 103 patients, the maximum number of cases were found to be positive by LAMP assay, that is, in 87 (84.5%)

patients, followed by culture positive in 78 (75.7%), IS6110 PCR in 74 (71.8%), and smear positive in 70 (67.9%) patients. Of the 83 smear positive and/or culture positive cases, LAMP detected 77 (92.77%) cases, and was found to be superior to IS6110 PCR, which could detect 69 (83.1%) cases; a concordance of 0.6 was obtained between the two tests using kappa statistics. **Conclusion:** Overall, LAMP was simple and efficacious for early diagnosis of smear positive, culture positive cases as well as for confirmation of smear negative, culture negative cases, and was found to be superior to IS6110 PCR. J. Clin. Lab. Anal. 27:272–276, 2013. © 2013 Wiley Periodicals, Inc.

Key words: *Mycobacterium tuberculosis*; pulmonary TB; 16S rRNA LAMP; IS6110

INTRODUCTION

Tuberculosis (TB) remains a major public health problem worldwide and is a leading cause of death in developing countries. Early diagnosis of *Mycobacterium tuberculosis* (MTB) in clinical samples is important not only for initiating treatment but also for curbing the disease transmission to others in the community (1). In developing nations, diagnosis of TB is restricted to microscopic examination of the sputum smear (2), as it is least expensive, simple, and relatively easy to perform, however, it suffers with low sensitivity and specificity (3). Culture, though the gold standard for diagnosis, takes 3–6 weeks for growth to occur in LJ media and 1–6 weeks in automated systems like BACTEC and MGIT 960. Molecular techniques, especially polymerase chain reaction (PCR), have been used by various investigators for early diagnosis of TB with promising results (4). There are various pro-

ocols available for the amplification of specific targets of MTB complex but all these methods require expensive laboratory infrastructure and sophisticated technical skills, which make them inaccessible to most of the laboratories in resource-poor countries.

Recently, loop-mediated isothermal amplification (LAMP) assay has been developed as a novel technique for nucleic acid amplification (5). LAMP can amplify DNA with high specificity and efficiency under isothermal conditions using six sets of primers that recognize eight

*Correspondence to: Dr. Sunil Sethi, Additional Professor, Department of Medical Microbiology, Post Graduate Institute of Medical Education and Research, Chandigarh, India. E-mail: sunilsethi10@hotmail.com

Received 20 December 2011; Accepted 4 February 2013

DOI 10.1002/jcla.21596

Published online in Wiley Online Library (wileyonlinelibrary.com).

distinct regions on the target sequence. Unlike PCR, it does not require a denatured DNA template and can be performed using a simple water bath, thus eliminating the need for a thermocycler. Large amount of DNA is generated in less than an hour, and a positive LAMP reaction can be visualized with naked eyes without the need of gel electrophoresis. LAMP is anticipated to become the dominant and most widely used molecular method of choice for rapid detection of MTB in clinical samples, especially in resource-poor settings (6); however, there is limited evidence of the applicability of LAMP from developing countries. The present study was hence carried out to evaluate MTB-specific in-house LAMP assay targeting the 16s rRNA of MTB complex (MTBC), and to compare it with *IS6110* PCR (most common and widely used nucleic acid amplification test in developing countries) and conventional techniques in suspected cases of pulmonary TB.

METHODS

A total of 133 sputum specimens were collected from patients attending the chest clinic of Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh, India, from March to December 2010, presenting with cough, fever, weight loss, breathlessness, chest pain, exudation, infiltration, and cavitations. The patients were divided into following groups: (A) smear positive and culture positive (S+C+) ($n = 65$); (B) smear positive but culture negative (S+C-) ($n = 5$); (C) smear negative but culture positive (S-C+) ($n = 13$); (D) smear negative and culture negative, which were clinically and radiologically confirmed (S-C-) ($n = 20$); and (E) controls ($n = 30$) suffering from respiratory diseases other than TB, such as bronchial asthma ($n = 9$), pneumonia ($n = 8$), chronic obstructive pulmonary disease ($n = 5$), interstitial lung disease ($n = 4$), lung carcinoma ($n = 2$), and sarcoidosis ($n = 2$), in whom TB was excluded on the basis of clinical, radiological, and microbiological tests. Informed consent was taken from all the patients and the study was approved by the Institute Ethics Committee of PGIMER, Chandigarh.

Sample Collection

Sputum samples were collected in sterile capped containers and were transported to the laboratory within an hour and were processed the same day.

Microscopy and Culture

Smears were prepared from sputum samples and stained by Ziehl Neelsen method. Samples were decontaminated by N-Acetyl-L-Cysteine Sodium Hydrox-

ide (NALC-NaOH) method and cultured on LJ media, MGIT 960, and identified according to standard methods (7).

DNA Extraction

DNA was extracted as described by van Embden et al., with minor modifications (8). Briefly, 200 μ l of the sample was suspended in 200 μ l of TE buffer (10 mM Tris Cl, 1 mM EDTA) in a microcentrifuge tube, and incubated in boiling water for 5–10 minutes, followed by snap cooling at -20°C for more than 20 minutes. Then, 40 μ l of lysozyme (20 mg/ml) was added and the tube was incubated overnight at 37°C . Next day, 5 μ l of proteinase K and 56 μ l of SDS were added in each tube and kept at 65°C for 1 hr. Further, 80 μ l of NaCl (5 M) and 64 μ l of CTAB/NaCl were added and again incubated at 65°C for 1 hr. Then, equal volume of chloroform:isoamyl alcohol (24:1) was added and was mixed by vortexing, followed by centrifugation at 10,000 rpm for 5 minutes at 4°C . The upper aqueous layer was separated and chloroform:isoamyl alcohol step was repeated again. The clear aqueous layer was separated and precipitated with chilled isopropanol (60% of total volume) at -20°C overnight. After centrifugation at 10,000 rpm for 15 minutes at 4°C , the supernatant was discarded and the pellet was resuspended in chilled ethanol, and centrifuged at 10,000 rpm for 5 minutes at 4°C . Pellets were air dried and then re-dissolved in 30 μ l of 1X TE buffer. The extracted DNA were then stored at -20°C , and used for the amplification assays within 48 hr.

IS6110 PCR

IS6110 PCR was carried out according to the standard method as described previously to look for specific DNA bands of 123 bp (AlphaImagerTM 3400, Protein-Simple, Santa Clara, CA) (9). *M. tuberculosis* H37RV was used as positive control, and PCR grade water was used as negative control.

LAMP Assay

LAMP assay in the present study targeted 16s rRNA gene of MTBC, using one set of each—inner primers, outer primers, and loop primers. The primers described by Pandey et al. were used: Forward Inner Primer (FIP)—CACCCACGTGTTACTCATGCAAGTCGAACGGA AAGGTCT, Backward Inner Primer (BIP)—TCGGGATAAGCCTGGACCACAAGACATGCATC CCGT, Forward Outer Primer (F3)—CTGGCTCAG GACGAACG, Backward Outer Primer (B3)—GCTCAT CCCACACCGC, Forward Loop Primer (FLP)—GTT

TABLE 1. Comparison of IS6110 PCR and LAMP Assay

Test	Positive	Sn. (95% CI)	Sp. (95% CI)	PPV (95% CI)	NPV (95% CI)
IS6110	74/103	71.8 (62.7–80.2)	100 (88.4–100)	100 (95.1–100)	61.2 (37.5–64.1)
LAMP	87/103	84.5 (76–90.8)	100 (88.3–100)	100 (95.8–100)	65.2 (49.7–78.6)

CI, confidence interval; NPV, negative predictive value; PPV, positive predictive value; Sn., sensitivity; Sp., specificity.

CGCCACTCGAGTATCTCCG, Backward Loop Primer (BLP)—GAAACTGGGTCTAATACCGG (10).

The reaction was carried out in a total volume of 25 μ l, containing 0.2 μ M each of F3 and B3, 1.6 μ M each of FIP and BIP, 0.8 μ M each of FLP and BLP, 20 mM Tris-HCl (pH: 8.8), 10 mM KCl, 10 mM (NH₄)₂SO₄, 9 mM MgSO₄, 1.4 mM dNTP, 0.8 M Betaine (Sigma-Aldrich, New Delhi, India), 8 U *Bst* DNA polymerase (New England Biolabs, Ipswich, MA), and 5 μ l of extracted DNA. To find the optimum time and temperature for LAMP assay, the reactions were carried out at 60–68°C for 35 to 90 minutes. A positive and negative control was included in each run. *M. tuberculosis* H37RV was used as internal positive control, and PCR grade water was used as negative control. LAMP testing was blinded to compare it with smear microscopy, culture, and PCR.

LAMP amplicons were directly detected with the naked eye by adding 0.1% SYBR Green I (Invitrogen, Carlsbad, CA) to the tube and observing the color of the solution under UV light. The solution turned green in the presence of a LAMP amplicon, while it remained orange when there was no amplification. For confirmation of the products, the amplicons were analyzed by 2% agarose gel electrophoresis.

Statistical Analysis

Although culture isolation of MTB is the gold standard for diagnosis, none of the methods of laboratory diagnosis has 100% sensitivity; so, outcome parameters like sensitivity, specificity, positive predictive value, and negative predictive value were calculated taking confirmed cases of TB (smear positive or culture positive or clinically confirmed TB, i.e., groups A–D) as reference standard using the standard formulas. The concordance between 16s rRNA LAMP and IS6110 PCR was calculated using Kappa statistics. The data were analyzed using the SPSS version 15.0 software.

RESULTS

The samples were considered to be culture negative if no growth was detected in both LJ and MGIT and culture positive if growth was detected in either LJ or MGIT. All the specimens were processed on the same day; the microscopy results were available within 24 hr and both the amplification assays were performed within 48 hr of DNA

extraction, thus obtaining the results within 96 hr. Of the 103 patients (Group A–D), 70 (67.9%) were found to be smear positive, 78 (75.7%) were culture positive, LAMP was positive in 87 (84.5%), and IS6110 could detect TB in 74 (71.8%) patients, thus LAMP detecting the maximum number of cases among these assays. Both IS6110 and LAMP were negative in all the 30 control patients (Group E), giving a specificity of 100% (95% confidence interval [CI], 88.4–100). The positive and negative predictive values of LAMP were 100% and 65.2%, respectively (Table 1). There were 83 S+ and/or C+ cases (i.e. groups A–C), of which 69 (83.1%) were detected by IS6110 PCR and 77 (92.77%) by LAMP assay. The sensitivity of LAMP was 98.4% (95% CI, 91.7–99.7) in S+C+ and 76.9% (95% CI, 46.2–94.7) in S-C+. In S+C-, LAMP was positive in three and IS6110 in one patient. In 20 patients who were clinically and radiologically diagnosed (S-C-), IS6110 PCR could detect five (25%) and LAMP detected 10 (50%) patients (Table 2). Clinically, all these cases showed good response to anti-TB drug therapy. The concordance among IS6110 and LAMP by using kappa statistics was found to be “approximately significant” ($k = 0.6$) (Table 3).

DISCUSSION

The present study evaluated a MTB-specific in-house LAMP assay targeting the 16s rRNA 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+ samples, which is in agreement with a multicentric study on MTB detection, which showed the feasibility of using LAMP in developing countries (11). 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 our results. However, the sensitivity was 48.8% in S-C+ sputum samples, which was lower than that found in the present study (76.9%). Similarly, a study carried out in Nepal has shown sensitivity of LAMP to be 96.1% in S+C+ and 85% in S-C+ samples, thus illustrating that LAMP can be used to provide confirmation of clinically suspected pulmonary TB cases where smear is negative (12). Another study by Pandey et al. in Nepal has shown the sensitivity of LAMP to be 100% and specificity to be 94.2% in culture positive samples (10). Kohan

TABLE 2. Group-Wise Comparison of IS6110 PCR and LAMP Assay

Group	IS6110					LAMP				
	Positive	Sn. (95% CI)	Sp. (95% CI)	PPV (95% CI)	NPV (95% CI)	Positive	Sn. (95% CI)	Sp. (95% CI)	PPV (95% CI)	NPV (95% CI)
Group A S+C+ (65)	60	92.3 (82.9–97.4)	100 (88.4–100)	100 (93.9–100)	85.7 (69.7–95.1)	64	98.4 (91.7–99.7)	100 (88.4–100)	100 (94.3–100)	96.7 (83.2–99.5)
Group B S+C- (5)	1	20 (0.5–71.2)	100 (88.4–100)	100 (2.5–100)	88.2 (72.5–96.7)	3	60 (15.4–93.5)	100 (88.4–100)	100 (30.5–100)	93.8 (79.2–99.5)
Group C S-C+ (13)	8	61.5 (31.6–86)	100 (88.4–100)	100 (62.9–100)	85.7 (69.7–95.1)	10	76.9 (46.2–94.7)	100 (88.4–100)	100 (68.9–100)	90.9 (75.6–97.98)
Group D S-C- (20)	5	25 (8.17–49.1)	100 (88.4–100)	100 (47.9–100)	66.7 (51–79.9)	10	50 (27.2–72.7)	100 (88.4–100)	100 (68.9–100)	75 (58.8–87.2)

CI, confidence interval; NPV, negative predictive value; PPV, positive predictive value; S+C+, smear positive, culture positive; S-C-, smear negative, culture positive; S-C-, smear negative, culture negative; Sn., sensitivity; Sp., specificity.

TABLE 3. Concordance Between IS6110 and LAMP for TB Confirmed Patients

N = 103	LAMP +	LAMP -	Kappa value
IS6110 +	74	0	0.6
IS6110 -	13	16	

N, number of patients.

et al. evaluated LAMP based on IS6110 comparing it with IS6110 PCR and found a sensitivity of 100% in culture positive samples for LAMP and 91.6% for PCR (13). Our results are in agreement with these studies, and reflect that 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.

In suspected cases, that is, S-C- but clinically and radiographically diagnosed, LAMP detected 50% of the cases, which is higher than IS6110 PCR (25%). All these patients were followed up after 2 months and they had shown good response to anti-TB therapy. These results are however in contrast to the study by George et al. in which LAMP did not appear to detect any additional true positives or cases that could not be detected by smear (14). According to their study, LAMP assay may be useful only in smear positive samples whereas our study clearly indicates that LAMP also has good sensitivity in smear negative cases. In S+C- but clinically confirmed cases, LAMP could detect three cases (60%) suggesting it to be highly sensitive in detection of small number of tubercle bacilli released from hidden foci in sputum.

The overall efficiency of LAMP (84.4%) in the present study was higher than IS6110 PCR (71.8%) in diagnosing the patients infected with MTB though substantial agreement was found between the two assays (kappa value = 0.6). A previous report where LAMP targeting IS6110 was compared with IS6110-based conventional PCR showed 20 times more sensitivity of LAMP over conventional PCR (15). The authors attributed this discrepancy to the fact that IS6110 is a repetitive sequence and not a single copy gene.

In conclusion, the present study demonstrated that LAMP assay is particularly efficient in cases of confirmed TB (S+C+), however, it can be used in establishing the diagnosis of suspected TB patients (S-C-) as well. Moreover, its performance was superior to IS6110 PCR, which is the most widely used amplification assay for diagnosis of MTB in developing countries. Thus considering the rapidity (results obtained within 96 hr), high sensitivity, specificity, and simplicity (does not require sophisticated equipment); LAMP is a molecular diagnostic test, which holds the potential to be introduced into the national TB control programs of developing countries, however,

it needs further evaluation and validation in more such studies at different centers so as to establish its usefulness at peripheral laboratories.

CONFLICT OF INTEREST

None to declare.

REFERENCES

1. Brodie D, Schluger NW. The diagnosis of tuberculosis. *Clin Chest Med* 2005;26:247–271.
2. Greenaway C, Menzies D, Fanning A, Grewal R, Yuan I, Fitzgerald MJ. Delay in diagnosis among hospitalized patients with active tuberculosis: Predictor and outcomes. *Am J Respir Crit Care Med* 2002;165:927–933.
3. Tiwari RP, Hattikudur NS, Bharmal RN, Kartikeyan S, Deshmukh NM, Bisen PS. Modern approaches to a rapid diagnosis of tuberculosis: Promises and challenges ahead. *Tuberculosis* 2007;87:193–201.
4. Cheng VCC, Yew WW, Yuen KY. Molecular diagnosis in tuberculosis. *J Clin Microbiol* 2005;24:711–720.
5. Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N. Loop mediated isothermal amplification of DNA. *Nucleic Acids Res* 2000;28:e63.
6. Neonakis IK, Spandidos DA, Petinaki E. Use of loop-mediated isothermal amplification of DNA for the rapid detection of *Mycobacterium tuberculosis* in clinical specimens. *Eur J Clin Microbiol Infect Dis* 2011;30:937–942.
7. Levin W, Brandon GR, Mc MS. The culture method of laboratory diagnosis of tuberculosis. *Am J Public Health Nations Health* 1950;40:1305–1310.
8. van Embden JDA, Cave MD, Crawford JT, et al. Strain identification of *Mycobacterium tuberculosis* by DNA fingerprinting: Recommendations for a standardized methodology. *J Clin Microbiol* 1993;31:406–409.
9. Eisenach KD, Cave MD, Bates JH, Crawford JT. Polymerase chain reaction amplification of repetitive DNA sequence specific for *Mycobacterium tuberculosis*. *J Infect Dis* 1990;161:977–981.
10. Pandey BD, Poudel A, Yoda T, et al. 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* 2008;57:439–443.
11. Boehme C, Nabeta P, Henostroza G, et al. Operational feasibility of using loop-mediated isothermal amplification for diagnosis of pulmonary tuberculosis in microscopy centers of developing countries. *J Clin Microbiol* 2007;45:1936–1940.
12. Aryan E, Makvandi M, Farajzadeh A, et al. Loop-mediated isothermal amplification (LAMP) for the direct detection of human pulmonary infections with environmental (nontuberculosis) mycobacteria. *Jpn J Infect Dis* 2009;62:212–214.
13. Kohan L, Shahhosseiny MH, Razavi MR, Parivar K, Moslemi E, Werngren J. Evaluation of loop mediated isothermal amplification for diagnosis of *Mycobacterium tuberculosis* complex in clinical samples. *Afr J Biotech* 2011;10:5096–5101.
14. George G, Mony P, Kenneth J. Comparison of the efficacies of loop-mediated isothermal amplification, fluorescence smear microscopy and culture for the diagnosis of tuberculosis. *PLoS One* 2011;6:e21007.
15. Aryan E, Makvandi M, Farajzadeh A, et al. A novel and more sensitive loop-mediated isothermal amplification assay targeting IS6110 for detection of *Mycobacterium tuberculosis* complex. *Microbiol Res* 2010;165:211–220.