Comparative Evaluation of Microscopy and Loop-Mediated Isothermal Amplification (Lamp) Assay for the Diagnosis of Tuberculosis

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Abstract

Tuberculosis (TB) is one of the major global health problem which affects millions of people each year. For the routine diagnosis of TB, microscopic technique is used but it has lower sensitivity and specificity. This study was carried out to evaluate the efficacy of loop mediated isothermal amplification (LAMP) over smear microscopy to detect *Mycobacterium tuberculosis*. Out of 84 processed samples processed in this study, the diagnostic tests showed varying results. Using smear microscopy, 16(19.05%) and with the LAMP assay, 17(20.24%) cases were found positive for *M. tuberculosis*. The sensitivity of the LAMP assay (87.5%) was greater than that of microscopy (82.35%) while the specificity of both methods was comparable (>95%). Being more sensitive than microscopy, LAMP assay is more likely to show the positive result and solve the errors in diagnosis of the TB cases. Thus, LAMP assay can be an important and cost-effective tool for appropriate and timely diagnosis of TB patients. This can further assist to implement intervention programs against TB.

Key words: *Tuberculosis, Global health problem, LAMP, Diagnostic tool, Microscopy*

Background

Tuberculosis (TB) is an age old disease and one of the major global health problems. Deterioration of public health programs aimed at preventing tuberculosis and encouraging completion of drug therapy for treatment of the disease is cited as a reason for the increasing rate of TB. Globally, approximately 10 million people are affected by this disease each year. As per the report from the last five years, the causative agent of this disease has been a leading cause of death from a single infectious agent, ranking above HIV/AIDS. This is despite the fact that, with a timely diagnosis and correct treatment, most people who develop TB disease can be cured. More than 95% of deaths due to TB occurred in developing countries out of which 50% were recorded from India, China, Indonesia, Pakistan, and the Philippines. In Nepal, about 45% of the total population is expected to have TB infection and the number of deaths due to tuberculosis is 5,000 to 7,000 people every year.

In most developing countries, microscopic examination of sputum is the widely available tool for routine diagnosisof TB. But the reliability of this method has been challenged because of lower sensitivity and specificity[4]. The lack of standardized procedures and trained microscopists on reading acid-fast bacilli (AFB) sputum smears present further drawbacks to finding cases in such countries. Culture, the gold standard diagnostic method, is highly sensitive but the slow multiplication and difficulty of selective cultivation often limits the culture based diagnosis[5]. Thus, n**ew technologies with higher sensitivity and specificity are urgently needed to confirm the clinical diagnosis and help reduce TB transmission.**

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The loop-mediated isothermal amplification (LAMP) assay – a nucleic acid amplification technology – was first proposed by Notomi[6]. Under isothermal condition, LAMP can amplify DNA with high specificity and efficiency using six sets of primers that recognize eight distinct regions on the target sequence. Unlike PCR, LAMP reaction does not require a denaturedDNA template and relies on auto cycling strand displacement DNA synthesis by a *Bst* DNA polymerase[7]. LAMP produces extremely large amounts of amplified products which enables simple visual detection and thus eliminates the need for electrophoresis.

Although bacterial culture is the gold standard for TB diagnosis, it is an expensive and slow technique and so, cannot be used for routine diagnosis. The LAMP assay, however, can be a simple, inexpensive and reliable choice for the TB diagnosis as this test combines rapidity of microscopy and sensitivity of bacterial cultural methods[9]. In the context of Nepal, the purpose of this study is to assess whether the LAMP assay can be a better and efficient method than microscopy. Additionally, the findings of this research can provide us a greater understanding of the prevalence rate of TB in terms of sex and age.

Material and Method

Sample collection and evaluation

In this study, a total of 84 samples were collected from the participants (February 2017 to October 2017) who were referred for a LAMP test and processed at Healthy Nepal, Balkhu. Informed consents were taken from the participants prior to the study. The study was approved by the Institutional Ethical Committee.

Sputum and different extra-pulmonary samples (urine, abscess, pleural fluid, peritoneal fluid, CSF and pericardial fluid) were the target samples of choice in this study. A series of at least three single specimens were collected initially (preferably on different days) from TB patients. First one on the spot, second early morning and third once again on the spot in which patients were instructed to take a deep breath and coughed deeply and vigorously[10]. Specimens were clearly labeled with patient's information and date of collection in a sterile plastic container.

Digestion, Decontamination and Concentration of Specimen

All other samples, except cerebrospinal fluid (CSF), pleural, peritoneal and pericardial fluid were decontaminated and homogenized with NALC-NaOH solution. Furthermore, for the purpose of digestion, decontamination and concentration of specimens, the protocols of Chatterjee et al were followed[11].

Acid-fast microscopy

The smear was heat fixed by placing the slide over flame three or four times with the smear on upper position and allowed to cool before staining. Further, smears were stained with Ziehl-Neelsen (Z-N) stain and examined for acid-fast bacilli (AFB) by microscopy. The positive organisms appeared as bright red against blue background.

DNA Extraction

DNA was extracted from the concentrated samples by alternate boiling and freezing method. Samples were heated at 95^0C for 10 minutes and freezed at -20^0C for 30 minutes for three times[13]. Thus, extracted DNA was stored in deep freeze until analysis.

LAMP Reaction

Altogether, six primers i.e. outer primer (F_3 and B_3), a forward inner primer (FIP), a backward inner primer (BIP) and loop primers (loop F and loop B) were used. They recognize eight distinct regions on 16S rRNA gene of the target DNA. LAMP was performed in a total volume of 25 µl reaction containing LAMP buffer, Betaine, $MgSo_4$, Primer mix (F_3, B_3, BIP, FIP, loop F, loop B), dNTPs (2.5 mM each), Bst DNA polymerase (8 units/ ml), 0.1% methyl green dye, DNA samples and distilled water. Constituents of master mixture (25µl/tube) were 16.5µl LAMP premix (LAMP buffer, Betaine, $MgSO_4$, primer mix), 1.5µl dNTPs (25mM), 1.0µl 0.1% Methyl green, 1.0µl Bst DNA polymerase and 5µl sample DNA. The whole reaction was performed at isothermal temperature (64^0C for one hour)[14]. A positive and negative control was included in each reaction.All the reagents, primers, and solutions required for LAMP were provided by Eiken Chemical Co. Ltd, Japan.

LAMP result

LAMP amplicons of positive organisms in the reaction tube were directly detected with the naked eye by observing the change in color of the solution containing reaction mixture with methyl green which stains DNA and in large amount of amplicons give

distinguished blue green color.

Disposal and Decontamination

The waste materials were first treated with 0.5% Sodium hypochloride and then autoclaved at 121^0C temperature for 15 minutes before disposal.

Statistical Analysis

All data obtained was statistically analyzed by using statistical package for social science version 20 software package. Sensitivity and specificity were compared using Chi-square test.

Results

Distribution of infection by microscopy and LAMP

Out of 84 clinical specimens processed, 19.05% (16/84) were microscopy positive, however, the same specimens were20.24% (17/84) positive with the LAMP assay.

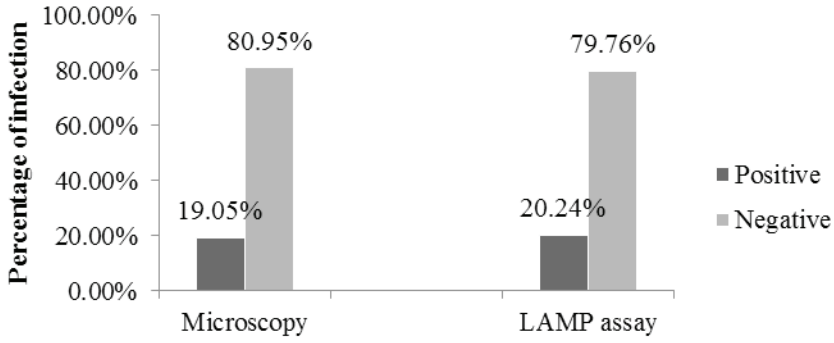

Fig: Distribution of infection by microscopy and LAMP

Sex-wise distribution of cases

In the sex-wise distribution of cases, 52 were male participants while 32 were female participants. Under microscopy and LAMP methods, male participants showed varying results while female participants had similar incidence of TB. With microscopy, 19.2% (10/52) male cases had positive infection while the rate was 21.1% (11/52) using LAMP. Similarly in the case of female participants, the incidence rate was 18.7% (6/32) for both microscopy and LAMP.

Table 1: Sex-wise distribution of cases

Gender	Total cases (%)	Microscopy		LAMP	
		Positive (%)	Negative (%)	Positive (%)	Negative (%)
Male	52 (100)	10 (19.2)	42 (80.8)	11(21.1)	41 (48.8)
Female	32 (100)	6 (18.7)	26 (81.2)	6 (18.7)	26 (81.2)

Age wise distribution of positive cases as detected by Microscopy and LAMP

The age-wise microscopic results shows that the infection was highest in the age-group 31-40 years 31.25% (5/16)), while lowest in the age-group <10 years (0%). With the LAMP assay, age-wise infection rate was highest in the age-group 31-40 years (29.4% (5/17)) and lowest in <10 years (0%).

Evaluation of LAMP with reference to microscopy

This showed 14 samples were positive by both microscopy and LAMP. With the LAMP assay, three microscopically negative cases were positive. Also with microscopy test, two LAMP assay negative cases were positive and 65 cases were negative by both tests. Sensitivity and specificity of the LAMP assay was 87.50% and 95.59% respectively. The predictive values of both of the tests were under the confidence interval.

Table 2: Evaluation of LAMP assay with microscopy (n = 84) at 95% CI

LAMP (2)	Microscopy (1)		Total	Sensitivity	Specificity	Positive Predictive value	Negative Predictive value
	Positive	Negative					
Positive	14	3	17	1=82.35% (64.2%-100%) 2=87.5% (71.3%-100%)	1=97.01% (92.9%-100%) 2=95.6% (90.7%-100%)	1=87.5% (71.3%-100%) 2=82.35% (64.2%-100%)	1=95.6% (90.7%-100%) 2=97.01% (92.9%-100%)
Negative	2	65	67				
Total	16	68	84				

Discussion

The main finding of this study shows that the LAMP assay can be a better and efficient method for tuberculosis diagnosis in Nepal. In this study, sensitivity of LAMP (87.5%) and microscopy (82.35%)are within in confidence interval. Similarly, specificity of LAMP (95.6%) and microscopy (97.01%) are also within the confidence interval. The sensitivity of the LAMP assay is consistent with other studies, but the lower specificity in contrast to microscopy (95.59%< 97.01%) might be due to human error during the laboratory process as most of the previous studies had recorded higher specificity of the LAMP assay[16,17,18]. In a similar study in Nepal, the sensitivity of MTB-LAMP in culture-positive samples was 100 % (96/96)[9]. In some other studies, the LAMP assay had lower sensitivity but high specificity[19,20]. The limitation of this study is lower sample size. This is because the subjects who were referred for a LAMP test (over a period of six months) were not many in number.

In our study, we found 20.24% (17/84) of the cases had positive infection. In Nepal, previous studies have also reported high prevalence of infection i.e. 27.98% (585/2091) in thirteen districts[21]. Also, studies in Western Nepal[22] showed a positive rate of TB around 10%. From these studies, we can infer that TB prevalence differs in Nepal depending on places and also sample size. Again, our study does not show a significant difference (not shown) in sex-wise prevalence (male=21.1% and female=18.7%). This means that both males and females may be equally exposed to the risk factors. Age-wise study showed people above 10 years are at higher risk of TB infection. In a similar study by Jaiswal et al [24] conducted in Western Nepal, they found 37.9% of TB infection in the age-group 15-35 years. Use of tobacco products [25] and extensive travelling by people (age>10 years) may expose them to TB infection.

According to the finding of our study, the LAMP assay provides a reliable and accurate choice for TB diagnostic testing in resource limited settings or where advanced PCR or cultural methods are not available. Being a rapid, inexpensive and technically not demanding diagnostic tool, this method can be used as a routine diagnostic tool. Furthermore, this can bring down the costs of diagnosis with complicated cases. This can also pave the way for clinicians in Nepal to prescribe

the right TB cases for DOTS program. Additionally, researches using this diagnostic test would increase the credibility and relevance in the context of Nepal.

When we compare the values of sensitivity, specificity, positive and negative predictive values of either test, it indicates microscopy can still be appropriate as a primary test. But the results of microscopy cannot be always relied upon. In this condition, application of the LAMP assay would greatly assist in the detection and confirmation of positive cases. In recent years, Nepal has been facing a lot of challenges to bring down the TB infection rate.The emergence of new cases often threatens the anti-TB program. The records of national tuberculosis programme also shows increasing incidences of TB in the country. One challenge for this may be inefficiency of the diagnostic methods being used. NTP has adopted END TB Strategy and aims to make TB control program accessible to people who need timely diagnosis and treatment for TB so that the epidemic condition of TB can be ended by 2030 [23]. Appropriate and timely diagnosis of TB victims is a must to implement intervention programs against TB.

Conclusion

In conclusion, this study showed that the LAMP assay could be better tool than microscopy for the diagnosis of tuberculosis. Microscopy can diagnose TB cases but cannot be a confirmatory test. The LAMP assay, however, could be a sensitive, fast and reliable tool and can be a confirmatory test for most of the cases. Thus this would help clinicians and health officers to prescribe the right TB cases for anti-tuberculosis drugs.

Competing Interest: The authors declare that they have no any competing interests. This research paper study been through original data without any third party interest. This paper targeted for related journal.

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