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Original articles

Loop-mediated isothermal amplification for detection of *Mycobacterium tuberculosis* and evaluation in paraffin-embedded lymph nodes

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ABSTRACT

Background: Tuberculosis is a treatable disease, but remains a major cause of mortality. Culture for *Mycobacterium* is needed to make a definitive diagnosis; thus, loop-mediated isothermal amplification (LAMP) was attempted for its detection in formalin-fixed, paraffin-embedded (FFPE) samples.

Materials and method: Archival FFPE blocks were used from 64 patients with a clinical diagnosis of tuberculous lymphadenitis from Srinagarind Hospital, Khon Kaen University, between 2007 and 2011. The clinical data, routine surgical pathologic examination, AFB stain, culture of *Mycobacterium* were researched retrospectively. Six primers were used to target the *M. tuberculosis* 16S rRNA gene for specific amplification and hydroxy naphthol blue (HNB) to detect the LAMP products.

Results: Seven of 23 (30%) samples of tuberculous lymphadenitis (culture positive for M. tuberculosis) were LAMP positive while all respective 23 and 21 cases of reactive hyperplasia lymph node (culture negative for *Mycobacterium*) and non-tuberculous lymphadenitis (culture positive for others *Mycobacterium*, excluding *M. tuberculosis*) were LAMP negative. Compared to culture, the sensitivity of LAMP in detecting *M. tuberculosis* in FFPE lymph node was 30.4%, specificity 100%, positive predictive value 100%, and negative predictive value 71.9%.

Conclusion: LAMP for diagnosis of tuberculous lymphadenitis in FFPE lymph nodes has limitations because of the degradation of *M. tuberculosis* DNA in paraffin-embedded samples. Its simplicity, quick-application, high specificity and low cost make it an alternative in fresh clinical samples. Further studies on fresh lymph nodes are warranted.

Keywords: Loop-mediated isothermal amplification, LAMP, *Mycobacterium tuberculosis*, tuberculous lymphadenitis

INTRODUCTION

Tuberculosis (TB) is a common chronic infectious disease caused by various strains of mycobacteria (*Mycobacterium tuberculosis* complex strains, e.g. *M. tuberculosis, M. microti, M. bovis, M. africanum*). *M. tuberculosis* is the most widespread infectious disease among humans [1]. TB causes an estimated 1.7 million deaths each year with more than 9 million new cases reported worldwide [2]. Typically, infection with *M. tuberculosis* manifests as pulmonary TB, but it can also affect other parts of the body in up to one-third of cases. Tuberculous lymphadenitis is the most common presentation of extra-pulmonary TB, particularly among HIV-infected patients [3].

A definitive diagnosis of TB is made by identifying *Mycobacterium* in a clinical samples; however, the difficult and time-consuming culture means that treatment is begun before cultures are confirmed [4]. Routine pathological diagnosis of tuberculous lymphadenitis depends on histopathology and detection of acid-fast bacilli using the Ziehl-Neelson stain (AFB stain). Among 89 (67 HIV-positive) tuberculous lymphadenitis-proven patients, histology and culture of a lymph node biopsy had the highest diagnostic yield (85% and 88%, respectively), followed by detection of acid-fast bacilli in biopsy smear (53%) [5]. A study among US immigrant patients (with culture positive tuberculous lymphadenitis samples) showed positive AFB staining in 44% (15/34 cases) [6].

In 2000, a novel technique that rapidly amplifies target DNA under isothermal conditions —the loop-mediated isothermal amplification (LAMP) method—was developed by Notomi [7]. LAMP employs a DNA polymerase with strand-displacement activity, along with two inner primers (FIP, BIP), outer primers (F3, B3) and loop primers (LF, LB), which recognize six separate regions within the target DNA [7]. The LAMP assay has a high specificity for *Mycobacterium* infection in fresh clinical samples [8-12]; however, no study of LAMP for *M. tuberculosis* of lymph node in FFPE samples has been conducted. We therefore planned a retrospective investigation of the potential use of LAMP as a simple assay for detection of *M. tuberculosis* using FFPE lymph nodes for pathological diagnosis.

MATERALS AND METHODS

Patients and tissue samples

Sixty-four patients who underwent lymphadenectomy and tissue culture for diagnosis of tuberculous lymphadenitis at Srinagarind Hospital (Khon Kaen University) between 2007 and 2011 were included. This retrospective study was approved by the Ethics Research Committee, Faculty of Medicine, Khon Kaen University (HE531403).

Archival FFPE lymph node blocks were obtained from the Pathology Department. Patient information (including laboratory results) was obtained from medical records. Detailed clinicopathological data are summarized in Table 1. Sixty-four lymph node specimens were divided into three groups, according to the results of culture for *Mycobacterium*: 1) the reactive hyperplasia lymph node (RHL) group had a negative culture for *Mycobacterium* (n=20), 2) the tuberculosis lymphadenitis (TBL) group had a positive culture for *M. tuberculosis* (n=23), and 3) the non-tuberculous lymphadenitis (NTL) group had a positive culture for *Mycobacterium* spp. but not for *M. tuberculosis* (n=21).

DNA extraction

Genomic DNA was extracted and purified from FFPE tissues using a QIAamp® DNA FFPE tissue kit (Qiagen, Germany). Ten-micrometer-thick paraffin sections were deparaffinized in xylene 2

Table 1 Clinico-pathological features of the 64 samples used for LAMP assay

Clinical data		**N (%)**
Sex	Male	26 (40.6%)
	Female	38 (59.4%)
Age (years)		6-69 yrs (42.57±16.04 yrs)
Immune status	Immuno-competent	43 (67.2%)
	Immuno-compromised (HIV infection : 8, Autoimmune disease: 12, Long term steroid use: 1)	21 (32.8%)
Had previous history of tuberculosis infection (Pulmonary TB: 5, Extrapulmonary TB: Lymph node: 12, Mediastinum: 1, Meninges: 2, Skin: 1, Spine: 1)		22 (34.4%)
Chest X-ray show cavitation		2 (3.1%)
Gross examination of lymph node	Number of lymph nodes	1-9 nodes (1.73±1.53 node)
	Diameter of lymph nodes	0.2-6 cm (1.28±0.82 cm)
Histological feature lymph node	Reactive hyperplasia (follicular hyperplasia or sinus histiocytosis)	28 (43.8%)
	Chronic granulomatous inflammation without caseous necrosis	12 (18.8%)
	Chronic granulomatous inflammation with caseous necrosis	20 (31.3%)
	Necrotic debrides without chronic granulomatous inflammation	4 (6.3%)
AFB staining	Positive	17 (26.6%)
	Negative	47 (73.4%)
Culture for Mycobacterium	No growth	20 (31.2%)
	Growth of *M. tuberculosis*	23 (35.9%)
	Growth of *Mycobacterium* spp. (*M. tuberculosis*, excluded)	21 (32.8%)

times and dehydrated twice with alcohol. These sections were rehydrated with alcohol 3 times. Tissue sections were scraped into a microcentrifuge tube and lysed under denaturing conditions with proteinase K at 56°C for 1 hr. The lysate was applied to the QIAamp MinElute column and the bound DNA eluted with 50 µL of ATE buffer. The DNA concentration and its purity were determined using a NanoVue™ spectrophotometer (GE healthcare, NJ, USA).

LAMP assay

Six primers recognizing distinct regions of the 16S rRNA gene of *M. tuberculosis* (Figure 1) were designed for specific amplification using the PrimerExplorer V3 software (Eiken Chemical; https://primerexplorer.jp/lamp3.0.0/index.html). A primer set was composed of outer primers F3 and B3, inner primers FIP and BIP, and loop primers FLP and BLP. LAMP reactions were performed in a 25-µL reaction mixture [13]; comprising: 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 8 mM $MgSO_4$, Tween 20 (0.1%), 1.6 M betaine, 1.4 mM deoxynucleoside triphosphate, 1.6 µmol each of the inner primers FIP and BIP, 0.2 µmol each of the outer primers F3 and B3, 0.8 µmol each of the loop primers FLP and BLP and 8 U Bst DNA polymerase (New England Biolabs). Different volumes of template DNA were tested but 7.5 µL DNA solution extracted was optimal. Double-distilled water was finally used to make up the volume to 25 µL. No template DNA was added in the negative control reaction. The mixture was incubated at 65°C in a heat block for 60 min then at 80°C for 5 min to terminate the reaction.

Detection of LAMP product

A LAMP positive reaction was determined by visual assessment of the amplification reaction indicated by the hydroxy naphthol blue (HNB) changing color from violet to sky blue [7,13] (Figure 2).

Statistical analysis

The statistical evaluation was performed using SPSSTM 17 (KKU license). The number and diameter in the LAMP positive and LAMP negative groups were compared using the T-Test. The other data were analysed using the Chi-square test. Statistical significance was set at $p < 0.05$.

Primers	Sequences
FIP	CACCCACGTGTTACTCATGCAAGTCGAACGGAAAGGTCT
BIP	TCGGGATAAGCCTGGACCACAAGACATGCATCCCGT
F3	CTGGCTCAGGACGAACG
B3	GCTCATCCCACACCGC
FLP	GTTCGCCACTCGAGTATCTCCG
BLP	GAAACTGGGTCTAATACCGG

Figure 1 The 6 primers recognizing distinct regions of the 16S rRNA gene of M. tuberculosis. A primer set comprised the outer primers (F3 and B3), the inner primers (FIP and BIP) and the loop primers (FLP and BLP).

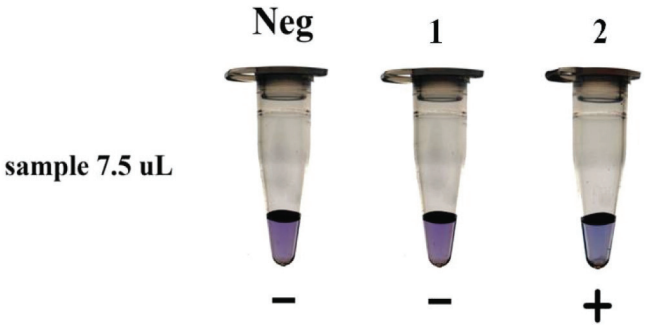

Figure 2 Visual examination of LAMP products (amplification amount of target DNA) by HNB, Neg (negative control); - (negative reaction, violet); + (positive reaction, sky blue); 1 and 2 (patient 1 and patient 2).

RESULTS

All FFPE samples yielded DNA extraction with different concentrations as determined by a spectrophotometer at O.D. 260 and tested with agarose gel (data not shown).

In the TBL group (n=23), 7 samples were LAMP-/AFB+ while 1 was LAMP+/AFB- and 9 were LAMP-/AFB- (Table 2). Seven of the TBL samples (7/23, 30.43%) were LAMP+ while all of the RHL group (0/20) and NTL group were LAMP-. Comparing these results to the cultures positive for M. tuberculosis, the respective sensitivity and specificity of LAMP was 30.4% and 100%. The respective positive (PPV) and negative (NPV) predictive values were 100% and 71.9% (p-value <0.001). Note that our design was only for LAMP detection of *M. tuberculosis*, so the sensitivity and specificity for a NTL group was not calculated. The AFB was negative in all of the cases of the RHL group (0/20) but positive in four of the cases of NTL group (4/21) and 13 of the TBL group (13/23). AFB had a sensitivity of 56.5%, a specificity of 90.2%, a PPV of 76.5%, and a NPV of 78.7%.

A LAMP+ had a significant correlation with: (a) the increased numbers of lymph nodes (p-value = 0.009), (b) the increased diameters of enlarged lymph nodes (p-value = 0.012), (c) chronic granulomatous inflammation with/without caseous necrosis (p-value = 0.045), (d) caseous necrosis seen in the histological examination (p value = 0.015), and (e) AFB staining (p-value <0.001) (Table 3).

Table 2 AFB staining and LAMP results in each lymph node study group.

	Reactive hyperplasia group (n=20)		Non-tuberculous lymphadenitis group (n=21)		Tuberculous lymphadenitis group (n=23)		
	AFB+	AFB -	AFB +	AFB -	AFB +	AFB -	Total
LAMP+	0	0	0	0	6	1	7
LAMP-	0	20	4	17	7	9	57

Table 3 LAMP results with clinical and laboratory data

Clinical data	p-value
Sex	0.899
Age	0.692
Immune status	0.80
Had previous history of tuberculosis infection	0.732
Chest X-ray show cavitation	0.61
Increased numbers of lymph nodes	0.009
Increase diameter of the enlarged lymph nodes	0.012
Chronic granulomatous inflammation with/without caseous necrosis	0.045
Caseous necrosis in histological examination	0.015
AFB staining	<0.001

DISCUSSION

In our study, LAMP had a positive correlation with the pathology of tuberculous lymphadenitis; viz., histological features, positive AFB staining, and the increased number and diameter of the nodes involved. These factors indirectly indicated that LAMP+ was related to an increased amount of *M. tuberculosis* in the sample. While DNA extraction from samples yielded a satisfactory amount of DNA, our LAMP assay had a high specificity but low sensitivity for detection of *M. tuberculosis* in FFPE lymph nodes. The LAMP assay usually has both a high sensitivity and specificity, because the amplification reaction occurs only when all six regions within a target DNA are correctly recognized by the primers. The low sensitivity of LAMP is possibly from bacterial DNA structural damage in some samples during tissue fixation and processing, and/or there may have been Bst polymerase inhibitor in the tissue during DNA extraction.

Studies of LAMP detection of *M. tuberculosis* in fresh clinical specimens samples—sputum and CSF yielded respective sensitivities of 100% and 88.23% [8,11]. The high sensitivity in these studies is possibly due to the well-preserved *M. tuberculosis* DNA in fresh samples. As an alternative approach for the diagnosis of TB in FFPE, other studies applied PCR for the detection of *M. tuberculosis* in FFPE samples for a sensitivity of up to 66% [14] and 78%, and a specificity of 88% [15].

Our retrospective study suggests that AFB with a sensitivity of 56.5% is acceptable for routine practice since it is less expensive and common practice; however, for confirming a diagnosis or handling problematic cases in FFPE, PCR would be more appropriate than LAMP. The LAMP technique—an assay with high specificity, simple to do and able to provide a diagnosis in less than 2 hours—is, however, suitable for rapid diagnosis in outpatient care, for field work and in areas where culture is not available.

CONCLUSION

LAMP for diagnosis of TB in FFPE tissue had a high specificity, PPV and NPV but a low sensitivity. The results of the current study and previous studies indicate that LAMP is suitable for the study of fresh specimens.

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Conflict of interest

None.

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