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Short Communication

Loop-Mediated Isothermal Amplification (LAMP) for the Direct Detection of Human Pulmonary Infections with Environmental (Nontuberculosis) Mycobacteria

Bal Ram Adhikari, Basu Dev Pandey[1]*, Prakash Ghimire, Bhawana Shrestha[2], Manoj Khadka, Tomoko Yoda[3], and Yasuhiko Suzuki[4]

Central Department of Microbiology, Tribhuvan University, Kathmandu; [1]Sukra Raj Tropical and Infectious Disease Hospital, Kathmandu; [2]German-Nepal Tuberculosis Project, Kathmandu, Nepal; [3]Osaka Prefectural Institute of Public Health, Osaka 537-0025; and [4]Hokkaido University Research Center for Zoonosis Control, Sapporo 001-0020, Japan

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SUMMARY: Most first-line anti-tuberculosis drugs have less in vitro activity against atypical mycobacteria. Loop-mediated isothermal amplification (LAMP) was used for the rapid diagnosis of mycobacterial species. The sensitivity of LAMP was 96.1% (49/51) in smear-positive and culture-positive sputum samples and 85.0% (17/20) in smear-negative and culture-positive samples. Of the 77 total LAMP-positive samples, 75 (97.4%) were identified as *Mycobacterium tuberculosis* and 2 (2.6%) as *M. intracellulare*. One of the *M. intracellulare*-infected cases was identified in a patient with suspected mycobacteriosis and another was found in a follow-up patient.

Tuberculosis (TB) is a major public health problem in Nepal. Approximately 45% of the total population is infected with TB, out of which 60% are adults. Every year, 40,000 people develop active TB, of whom 20,000 have infectious pulmonary disease. Though the introduction of treatment by a directly observed treatment short course (DOTS) has already reduced the number of deaths, 6,000 people annually continue to die of this disease. The current goal is to diagnose 70% of new infectious cases and to cure 85% of these patients, which may prevent approximately 50,000 deaths over the next 5 years (1). However, it is of interest to address the problem of the 15% of cases not cured by DOTS. They may be cases of multidrug resistance or atypical mycobacteria not responding to the DOTS therapy. Environmental (nontuberculosis) mycobacteria have been documented to be involved in human pulmonary infections in both developed and developing countries (2-6). Incidence rates ranging from 2 - 20% for atypical mycobacteriosis have been reported from various parts of the world (2,7,8).

The routine diagnosis method for TB by acid-fast smear is not effective for diagnosis of nontuberculous mycobacteria (NTM) infection, which requires either a biochemical test or molecular techniques. The conventional biochemical tests for identification of mycobacterial species are tedious and time-consuming because of the slow growth of mycobacteria on culture media (9). In addition to the widely used PCR, several other methods of nucleic acid amplification have been used for *Mycobacterium tuberculosis* detection (10-13). Loop-mediated isothermal amplification (LAMP) is a novel nucleic acid amplification method that enables the detection of trace amounts of DNA under isothermal conditions, namely at 64°C (15). LAMP showing high amplification efficiency has been used for the diagnosis of several diseases (12,14,17,18).

This study was carried out from November 2005 to October 2006 in Kathmandu. Samples were collected and examined by acid-fast smear and bacterial culture at the National TB Reference Laboratory of the German-Nepal Tuberculosis Project (GENETUP). DNA extraction was performed at the same place, and LAMP reactions were carried out at the Everest International Clinic and Research Center (EICRC). One hundred thirty sputum specimens (69 specimens from patients with suspected pulmonary mycobacteriosis and 61 from follow-up patients), requested for culture by physician, were obtained from GENETUP. After decontamination and concentration by N-acetyl L-cysteine-NaOH treatment, specimens were treated for LAMP, as described previously (18). The same concentrated specimens were subjected to culture and fluorochrome-staining. LAMP was performed by using a set of specific primers for *M. tuberculosis*, *M. avium*, *M. intracellulare*, and *M. kansasii* at 64°C for 1 h in a thermal cycler. A total of six primers recognizing eight distinct regions on the target DNA, termed outer primers (F3 and B3), a forward inner primer (FIP), a backward inner primer (BIP), and loop primers (loop F and loop B), as presented in Table 1, were used for each species.

The reaction was performed in a 25-μl reaction mixture consisting of a 2.5-μl 10 × LAMP buffer (200 mM Tris-HCl [pH 8.8], 100 mM KCl, 100 mM NH_4SO_4, and 1% Triton X-100), 14 mM dNTPs, 0.8 M betaine, 300 mM $MgSO_4$, 30 pmol each of BIP and FIP primers, 5 pmol each of FL and BL primers, 20 pmol each of F3 and B3 primers, 8U *Bst* DNA polymerase (New England Biolabs, Inc., Ipswich, Md., USA), 4 μl DNA samples, and Loopamp Fluorescent Detection Reagent (Eiken Chemical Co., Ltd., Tokyo, Japan). Loopamp Fluorescent Detection Reagent enables the direct detection of DNA amplification in reaction tubes by the naked eye as green fluorescence under ultra-violet light.

The specificity of the LAMP primers was examined by LAMP reaction on DNA mechanically extracted (11) from various mycobacterial strains, including *M. tuberculosis*, *M. bovis* Ravenel, *M. bovis* BCG, *M. africanum*, *M. microti*, *M. kansasii*, *M. avium*, *M. intracellulare*, *M. shimoidei*, *M.*

*Corresponding author: Mailing address: Sukra Raj Tropical and Infectious Disease Hospital, Teku, Kathmandu, Nepal. Tel: +977-1-4253395, E-mail: basupandey@wlink.com.np

Table 1. Primers used in this study

Target species	Primer name	Nucleotide sequence
M. tuberculosis	FIP	CACCCACGTGTTACTCATGCAAGTCGAACGGAAAGGTCT
	BIP	TCGGGATAAGCCTGGACCACAAGACATGCATCCCGT
	FL	GTTCGCCACTCGAGTATCTCCG
	BL	GAAACTGGGTCTAATACCGG
	F3	CTGGCTCAGGACGAACG
	B3	GCTCATCCCACACCGC
M. avium	FIP	TGCCCACGTGTTACTCATGCAAGTCGAACGGAAAGGCCT
	BIP	TCGGGATAAGCCTGGACCAGAAGACATGCGTCTTGA
	FL	GTTCGCCACTCGAGTACCTCCG
	BL	GAAACTGGGTCTAATACCGG
	F3	CTGGCTCAGGACGAACG
	B3	GCCCATCCCACACCGC
M. intracellulare	FIP	TGCCCACGTGTTACTCATGCAAGTCGAACGGAAAGGCCC
	BIP	TCGGGATAAGCCTGGACCTAAAGACATGCGCCTAAA
	FL	GTTCGCCACTCGAGTACCCCCG
	BL	GAAACTGGGTCTAATACCGG
	F3	CTGGCTCAGGACGAACG
	B3	GCCCATCCCACACCGC
M. kansasii	FIP	TGCCCACGTGTTACTCATGCAAGTCGAACGGAAAGGTCT
	BIP	CCGGGATAAGCCTGGACCACAAGGCATGCGCCAAGT
	FL	GTTCGCCACTCGAGTGTCTCCG
	BL	GAAACTGGGTCTAATACCGG
	F3	CTGGCTCAGGACGAACG
	B3	GCCCATCCCACACCGC

Table 2. LAMP sensitivity and specificity

Group	% Sensitivity (no. of LAMP-positive samples/no. of smear- and LJ-positive samples)	% Sensitivity (no. of LAMP-positive samples/no. of smear-negative, LJ-positve samples)	% Specificity (no. of LAMP-negative samples/no. of LJ-negative samples)
Suspected mycobacteriosis	100 (32/32)	87.5 (7/8)	86.2 (25/29)
Follow-up case	89.5 (17/19)	83.3 (10/12)	76.7 (23/30)

nonchromogenicum, *M. xenopi*, *M. scrofulaceum*, *M. gordonae*, *M. chelonae*, and *M. fortuitum*, found not to have a cross reaction. The sensitivity of LAMP was 100% (32/32) in smear-positive and culture-positive sputum samples and 87.5% (7/8) in smear-negative and culture-positive sputum samples from suspected TB patients. The specificity in culture-negative samples was 86.2% (25/29). Similarly, the sensitivity of LAMP was 89.5% (17/19) in smear- and culture-positive sputum specimens and 83.3% (10/12) in smear-negative, culture-positive specimens from follow-up patients. The specificity in the culture-negative samples was 76.7% (23/30). Out of 43 LAMP-positive samples from suspected mycobacteriosis patients; one was identified as *M. intracellulare*. Similarly, out of 34 LAMP-positive samples from the follow-up patients, one was identified as *M. intracellulare*. Both cases were found in males over age 50. Data from this study confirm the involvement of environmental mycobacteria in human pulmonary infection, as indicated in earlier reports by Allanana et al. (16).

In most parts of Nepal, diagnosis of TB is generally based on clinical symptoms such as fever, weight loss, persistent productive cough, hemoptysis, and an abnormal chest X-ray. The presence of acid-fast bacilli in sputum and X-ray abnormalities are not sufficient to establish a case of classical pulmonary TB, and identification of the mycobacterium isolate is necessary. A test that combines the rapidity of microscopy and the sensitivity of bacterial culture methods is necessary for the rapid diagnosis of mycobacteria at the species level. LAMP can be used for this purpose, providing results within 1 h with high sensitivity.

A multicenter study of *M. tuberculosis* detection showed the feasibility of using the LAMP method in developing countries (17). The study evaluated a prototype LAMP assay targeting the *gyrB* gene and using a simplified manual DNA extraction method. The sensitivity of MTB-LAMP was 97.7% (173/177) in smear-positive and culture-positive sputum samples and 48.8% (21/43) in smear-negative and culture-positive sputum samples. Similarly, another study by Pandey et al. on Nepalese patients showed the feasibility of LAMP for the detection of *M. tuberculosis*; the sensitivity of LAMP was 100% (96/96) in culture-positive sputum samples, and the specificity was 94.2% (98/104) in culture-negative sputum samples (18). In our present study, the sensitivity of LAMP was 96.1% (49/51) in smear-positive and culture-positive sputum samples, and the sensitivity was 85.0% (17/20) in smear-negative and culture-positive samples. The sensitivity obtained from this study is thus comparable to that reported for the above study.

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