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Rapid Multiplex Loop-Mediated Isothermal Amplification (m-LAMP) Assay for Differential Diagnosis of Leprosy and Post–Kala-Azar Dermal Leishmaniasis

Shweta Joshi,[1] Keerti K. Dixit,[1] Vanila Sharma,[1] V. Ramesh,[2] Ruchi Singh,[1]* and Poonam Salotra[1]*

[1]*Molecular Parasitology Laboratory, ICMR-National Institute of Pathology, Safdarjung Hospital Campus, New Delhi, India;* [2]*Department of Dermatology, Safdarjung Hospital, New Delhi, India*

Abstract. Leprosy and post–kala-azar dermal leishmaniasis (PKDL) are co-endemic neglected tropical diseases often misdiagnosed because of close resemblance in their clinical manifestations. The test that aids in differential diagnosis of leprosy and PKDL would be useful in endemic areas. Here, we report development of a multiplex loop-mediated isothermal amplification (m-LAMP) assay for differential detection of *Mycobacterium leprae* and *Leishmania donovani* using a real-time fluorometer. The m-LAMP assay was rapid with a mean amplification time of 15 minutes, and analytical sensitivity of 1 fg for *L. donovani* and 100 fg for *M. leprae*. The distinct mean Tm values for *M. leprae* and *L. donovani* allowed differentiation of the two organisms in the m-LAMP assay. Diagnostic sensitivity of the assay was evaluated by using confirmed cases of leprosy ($n = 40$) and PKDL ($n = 40$) (tissue and slit aspirate samples). All the leprosy and PKDL samples used in this study were positive by organism-specific QPCR and loop-mediated isothermal amplification assays. The diagnostic sensitivity of the m-LAMP assay was 100% (95% CI: 91.2–100.0%) for detecting PKDL and 95% for leprosy (95% CI: 83.1–99.4%). Our m-LAMP assay was successfully used to detect both *M. leprae* and *L. donovani* in a patient coinfected with leprosy and macular PKDL. The m-LAMP assay is rapid, accurate, and applicable for differential diagnosis of leprosy versus PKDL, especially in endemic areas.

INTRODUCTION

Leprosy, caused by *Mycobacterium leprae*, is a slow-progressing skin disease that may remain subclinical between the infection and clinical manifestations. This subclinical state may eventually lead to a systemic phase, predisposing nerve damage and subsequent deformities affecting skin tissues, nose, and eyes.[1] India has achieved the prevalence rate (PR) of 0.84/10,000 individuals in 2006 as a consequence of control measures taken under the National Leprosy Elimination Program (NLEP).[2] However even after continued efforts, the NLEP annual report (2018) suggests that the disease remains a constant peril with an approximate PR of 0.67/10,000 individuals and 126,164 new cases reported in 2017–2018.[3] The most likely cause of the high incidence rate of leprosy is the transmission caused by subclinical infections and delay in the diagnosis.

Visceral leishmaniasis (VL) caused by *Leishmania donovani* is another tropical disease that largely affects people living in poverty in developing countries and presents a severe threat to socioeconomic development.[4] In the Indian subcontinent, a dermal sequel of VL called as post–kala-azar dermal leishmaniasis (PKDL) appears in up to 15% of VL patients within 2–3 years of treatment.[5] In VL-endemic areas, active PKDL cases act as a durable reservoir of *L. donovani*, especially during the interepidemic period; therefore, identification of PKDL cases is crucial to control leishmaniasis.[6]

Leprosy can have a monomorphic or polymorphic presentation like PKDL, and rarely, in PKDL, nerves can also be involved histopathologically, although sensations are preserved on clinical examination.[7–9] Leprosy is diagnosed either by observing bacilli in Ziehl–Neelsen–stained slides prepared from slit skin smears or by histopathological analysis of lesions. These examinations have low sensitivity and specificity, are time consuming, and require technical expertise. Likewise, diagnosis of PKDL mainly relies on histopathology as gold standard. Serological tests, such as the rK39 strip test, are usually positive but are of limited value because of antibodies persisting from the past episode of VL. Molecular assays based on DNA amplification have been explored and developed for leprosy and PKDL for detection and quantification of causal organisms *M. leprae* and *L. donovani*.[10–16] However, the requirement for expensive equipment and elaborate experimental procedure forestalls the utilization of nucleic acid amplification assays in field settings.

Cost-effective isothermal amplification–based techniques, such as nucleic acid sequence–based amplification, strand displacement amplification, loop-mediated isothermal amplification (LAMP), and helicase-dependent amplification, can be applied as a substitute to expensive molecular methods.[17] Among these isothermal amplification methods, LAMP is a promising candidate. Here, in this study, we have applied LAMP for the differential diagnosis of PKDL versus leprosy with substantial sensitivity and specificity to avoid misdiagnosis. In the present study, we propose to develop a multiplex loop-mediated isothermal amplification (m-LAMP) assay for the differential diagnosis of PKDL and leprosy using real-time fluorometer Genie II (Optigene, Horsham, United Kingdom). Several LAMP-based studies have used Genie II that displays real-time amplification and amplicon annealing temperature (Tm) for sample detection.[18,19] Furthermore, the real-time fluorimetry LAMP (RealAmp) has been reported to be cost-effective; for instance, in diagnosis of malaria, the cost of running per sample was cheaper on the RealAmp platform using in-house buffer than with nested polymerase chain reaction ($2.66 versus $3.67).[20]

MATERIALS AND METHODS

Clinical samples. The study was conducted after obtaining ethical clearance under the guidelines of the Ethics Committee of the Safdarjung Hospital, New Delhi, India. Patients

* Address correspondence to Ruchi Singh or Poonam Salotra, ICMR-National Institute of Pathology, Safdarjung Hospital Campus, New Delhi 110029, India. E-mails: ruchisp@gmail.com or poonamsalotra@hotmail.com

presenting with characteristic clinical manifestations of leprosy or PKDL who reported at the Department of Dermatology, Safdarjung Hospital, New Delhi, were included in the study. All the leprosy and PKDL cases were confirmed by histopathology/microscopy and QPCR.[10,21]

At the pretreatment stage, 3-mm punch tissue samples and slit skin aspirates were collected in 200 μL NET buffer (150 mmol/L NaCl, 15 mmol/L Tris-HCl [pH 8.30], and 1 mmol/L EDTA) from hypopigmented skin lesions of 20 leprosy patients, which included 18 multibacillary (MB) and two paucibacillary (PB) cases. Presence of up to five lesions including truncal nerve thickening was classified as PB and more than that as MB.

Likewise, 3-mm punch tissue samples were collected from 20 PKDL patients, inclusive of seven nodular, seven papular, and six macular cases. Slit skin aspirate was collected from another 20 PKDL cases, which included eight nodular, eight papular, and four macular patients (Table 1). Punch tissue samples from patients with other skin diseases (n = 10), including cutaneous tuberculosis, vitiligo, sporotrichosis, pityriasis lichenoides chronica, lichen sclerosis, and pityriasis rosea, along with slit aspirates from normal skin (n = 10) were included as controls. For DNA isolation, QIAamp DNA mini kit (Qiagen, Hilden, Germany) was used following the manufacturer's instructions. The isolated tissue DNA was eluted in 50 μL (≈170 ng/μL) and slit aspirate DNA in 20 μL (≈3.5 ng/μL) of nuclease-free water and stored at −30°C until further use.

A rare case of an 18-year-old woman with hypopigmented macules on the body hailing from an endemic area of leprosy and PKDL came to the Department of Dermatology, Safdarjung Hospital. She was diagnosed as leprosy positive based on histopathology and Zeihl–Neelsen staining microscopy. Coinfection of macular PKDL was suspected on the basis of the rK39 strip test and confirmed by QPCR.[10] Her pretreatment slit-skin aspirate was collected for the m-LAMP assay to check if the assay was capable of detecting *M. leprae* and *L. donovani* in the coinfected clinical sample.

Pathogen DNA samples. DNA was extracted from *L. donovani* AG83 (MHOM/IN/83/AG83), *Mycobacterium tuberculosis*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Plasmodium vivax*, and *Plasmodium falciparum*. *Mycobacterium leprae* genomic DNA was obtained from Biodefense and Emerging Infections Research Resources Repository, BEI Resources, Manassas, VA (NR-19350).

Optimization of multiplex LAMP reaction. For the present study, LAMP assay was performed in a portable real-time fluorometer (Genie II). This instrument runs the isothermal amplification method to detect the target by fluorescence measurement and melting curve analysis and displays real-time amplification and amplicon annealing temperature indicated by Tm. The settings involve the use of a precision LED-based optical detection system, which monitors all dyes with excitation at 470 nm and detection above 510 nm.

A set of six LAMP primers, including two outer primers (forward primer F3 and backward primer B3), two inner primers (forward inner primer FIP and backward inner primer BIP), and two loop primers (forward loop primer [FLP] and backward loop primer [BLP]), targeting the kinetoplast minicircle sequence (kDNA) present in *L. donovani* were selected for the m-LAMP assay.[22] The primers for *M. leprae* targeting the repetitive and specific RLEP gene sequence uniquely present in *M. leprae* were designed such that the annealing temperature of the amplified product was distinct from that of *Leishmania*, thus making them suitable for multiplexing (Table 2). Individual LAMP assays were also run in Genie II for determination of the limit of detection and distinct Tm values of *M. leprae* and *L. donovani*.

The m-LAMP assay was initially run at 65°C temperature with different combinations of primer mixes (1X kDNA + 1X RLEP; 1X kDNA + 0.5X RLEP; 0.5X kDNA + 1X RLEP; or 0.5X kDNA + 0.5X RLEP) and serially diluted genomic DNA of *M. leprae* and *L. donovani* (1 ng/μL to 1 fg/μL) to determine the shortest amplification time and highest fluorescence reading. The amplification was performed in triplicate and represented as fluorescence first detected, and Tm (melting temperature) values were determined by the melting curve analysis. A no-template control was used in each experiment, in which nuclease-free water was added instead of template DNA.

Briefly, the reaction was carried out in a final reaction volume of 15 μL containing 9 μL of Isothermal Master Mix ISO-001 (Optigene) (containing *Geobacillus* species DNA polymerase, thermostable inorganic pyrophosphatase, optimized buffer including $MgCl_2$, dNTPs, and ds-DNA dye), 3 μL primer mix consisting of six primers each for *M. leprae* and *L. donovani* (F3 and B3 primers at 5 picomole, FIP and BIP primers at 20 picomole, and FLP and BLP primers at 10 picomole final concentration), 1 μL DNA (*M. leprae* or *L. donovani*), and nuclease-free water to make up the volume. The LAMP assay was run at 65°C for 40 minutes with a melting curve analysis step for specificity with an annealing curve from 98°C to 80°C ramping at 0.05°C per/second. Once the assay was established, it was applied to tissue and slit-skin aspirate samples of leprosy and PKDL patients combined randomly to determine diagnostic sensitivity of the assay. Tissue DNA (1 μL) of a leprosy sample was pooled with tissue DNA (1 μL) of a PKDL sample in the m-LAMP reaction mix. Similarly, DNA samples

TABLE 1

Profile and clinical categorization of leprosy and PKDL patients included in the study

Demographic details				
	Gender (*N*)		Age range (years)	
Disease	Male	Female	Male	Female
Leprosy (*N* = 20)	13	7	24–70	17–50
PKDL-tissue biopsy (*N* = 20)	15	5	16–42	16–40
PKDL-slit aspirate (N = 20)	15	5	12–40	15–40

Clinical categorization		
Leprosy	Multibacillary	18
Clinical classification	Paucibacillary	2
BI	0 to 6+	–
Ridley–Jopling classification (histopathology)*	BT	6
	BB	2
	BL	8
	LL	4
PKDL – tissue biopsy	Macular	6
Clinical presentations	Papular	7
VL history	Nodular	7
	10 months–25 years	–
PKDL- slit aspirate	Macular	4
Clinical presentations	Papular	8
VL history	Nodular	8
	1–20 years (No history in two cases)	–

BB = borderline; BI = bacterial index; BT = borderline tuberculoid; LL = lepromatous; VL = visceral leishmaniasis. BT = 0–2+; BB = 2–5+; BL = 4–5+; LL = 5–6+.
* As per Ridley–Jopling classification given on the basis of BI.

TABLE 2

Primers designed for amplification of *Mycobacterium leprae* RLEP gene (accession no. X17153.1) and *Leishmania donovani* kinetoplast minicircle sequence (accession no. Y11401) for the m-LAMP assay

Loop-mediated isothermal amplification primer	Primer sequence (5′-3′)	Reference
M. leprae		
RLEP-F3	TTGTTGGTGGGTGGCTGA	Unpublished data
RLEP-B3	CGGCGCTAACAACTATCCTC	
RLEP-FIP	TTACGTGCGCCGCGCTAATCCTGCTTTCGATGAGGCTTCG	
RLEP-BIP	GGTGGATGCTGCTTGGTCTACATGCATCGATATCGCCTTCAG	
RLEP-FL	CACTGCGGCAAAGCACA	
RLEP-BL	TGTTGATGATGCCAGGGGC	
L. donovani		
F3	GGTGCAGAAATCCCGTTCAA	13
B3	CAACCCCCAAACCCCAAG	
FIP	CCCCTCCACCCGACCCTATTTAAATGCCAAAAATCGGCTCC	
BIP	TCGGGGCTCGGACGTGTGTCCAGGTCCAAAACCCCATAC	
FLP	CACCAACCCCCAGTTTCCCG	
BLP	GGGGACTTTGGAGTGGGTTGTA	

isolated from slit-skin aspirates of leprosy and PKDL (1 µL each) were pooled.

RESULTS

Analytical sensitivity of m-LAMP. The optimized m-LAMP assays were run at 65°C for 40 minutes using the 0.5X kDNA + 0.5X RLEP primer mix. Analytical sensitivity of the m-LAMP assay was determined by testing in triplicate serial dilutions of *L. donovani* and *M. leprae* genomic DNA on three separate days to establish reproducibility of the assay for differential rapid detection of PKDL and leprosy on the Genie II fluorometer. For *L. donovani*, the detection limit was 1 fg equivalent to less than one parasite,[23] with amplification time

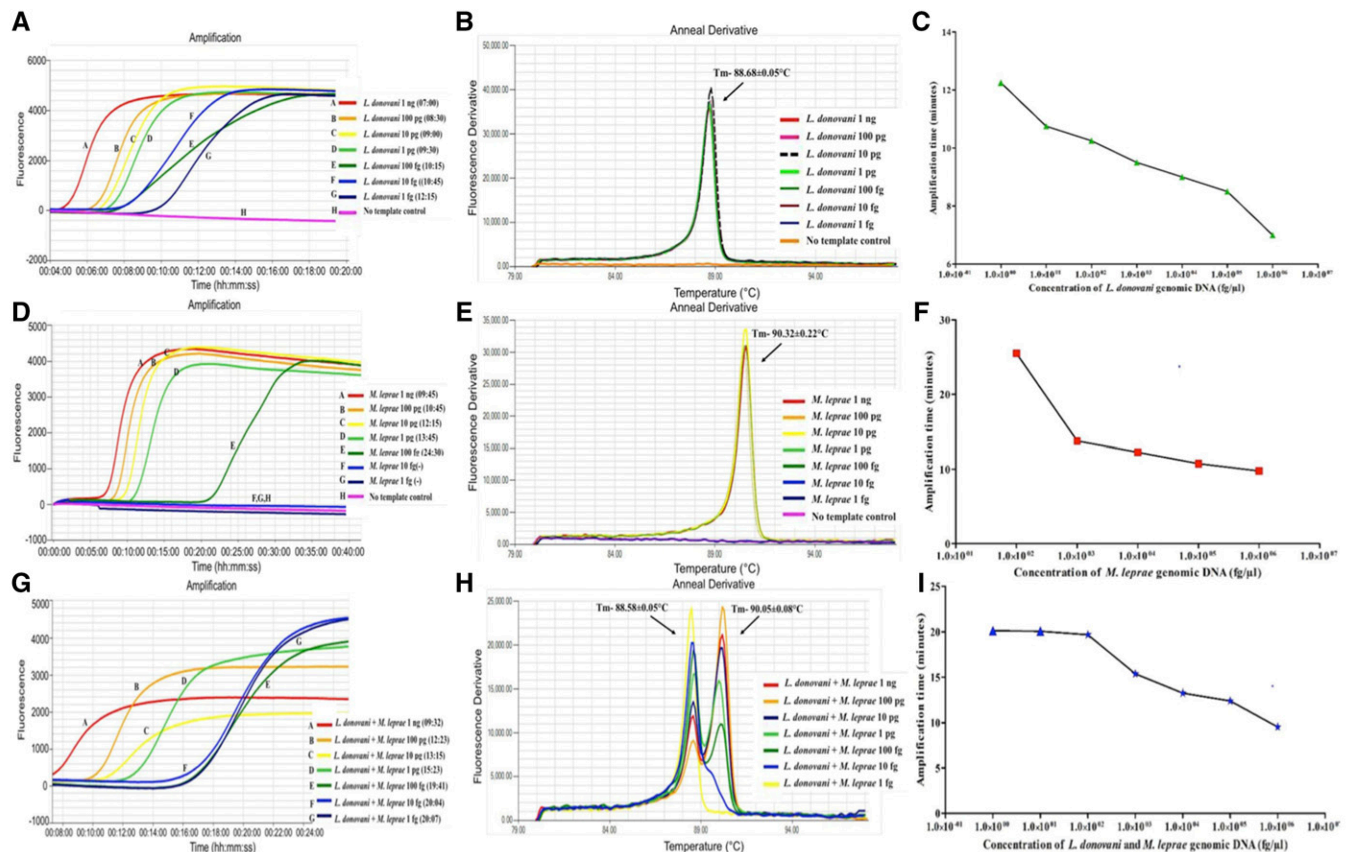

FIGURE 1. Sensitivity of multiplex loop-mediated isothermal amplification in Genie II for the detection of *Mycobacterium leprae* and *Leishmania donovani*. Ten-fold serial dilution of *M. leprae* and *L. donovani* genomic DNA was tested. (**A**) Amplification curve for *L. donovani* generated from 1 ng/µL to 1 fg/µL shows time on the *x*-axis and fluorescence on the *y*-axis. (**B**) Melting peak at 88.68 ± 0.05°C generated by the melting curve analysis. (**C**) Amplification time (minutes) vs. concentration of genomic DNA (fg/µL) for *L. donovani*. (**D**) Amplification curve for *M. leprae* generated from 1 ng/µL to 100 fg/µL. (**E**) Melting peak at 90.32 ± 0.22°C. (**F**)Amplification time (minutes) vs. concentration of genomic DNA (fg/µL) for *M. leprae*. (**G**) Combined amplification curve (of *M. leprae* and *L. donovani*) generated from 1 ng/µL to 1 fg/µL. (**H**) Two distinct melting peaks (88.58 ± 0.05°C for *L. donovani* and 90.05 ± 0.08°C for *M. leprae*) were generated by the melting curve analysis. (**I**) Amplification time (minutes) vs concentration of combined genomic DNA (fg/µL) of *L. donovani* and *M. leprae*. In total, 10 fg/µL and 1 fg/µL were only detected for *L. donovani* at 20.06 minutes and 20.11 minutes, respectively (time points depicted by Δ in the graph). This figure appears in color at www.ajtmh.org.

ranging from 7:00 minutes for 1 ng/μL to 12:15 minutes for 1 fg/μL and a mean Tm of 88.68 ± 0.05°C (Figure 1A–C). The detection limit of *M. leprae* was found to be 100 fg, which is equivalent to 30 bacilli.[24] The amplification time ranged from 9: 13 minutes for 1 ng/μL to 20:34 minutes for 100 fg/μL of *M. leprae* genomic DNA with a mean Tm of 90.32 ± 0.22°C (Figure 1D–F). When combined, the mean amplification time was 15:23 minutes (range 9:32 minutes to 20:07 minutes) with a mean Tm for *L. donovani* as 88.58 ± 0.05°C and for *M. leprae* as 90.05 ± 0.08°C (Figure 1G–I). The distinct values of Tm allowed the easy identification of PKDL and leprosy.

Identification of *M. leprae* and *L. donovani* by m-LAMP in clinical samples. Individually, all 20 PKDL and 20 leprosy tissue samples were positive in the LAMP assay. Similarly, all slit aspirate samples were also positive in their respective LAMP assay. When pooled, 20/20 PKDL (sensitivity 100%; 95% CI: 83.2–100.0%) and 20/20 leprosy (sensitivity 100%; 95% CI: 83.2–100.0%) tissue samples were positive in the m-LAMP assay. Similarly, in pooled slit aspirate samples of PKDL with leprosy, all 20/20 PKDL (sensitivity 100%; 95% CI: 83.2–100.0%) and 18/20 leprosy (sensitivity 90%; 95% CI: 68.3–98.8%) samples were positive. Based on the results obtained with tissue and slit samples, the m-LAMP assay differentially detected 100% PKDL (40/40; 95% CI: 91.2–100.0%) and 95% leprosy (38/40; 95% CI: 83.1–99.4%) samples as indicated by distinct Tm values (Figure 2A), although the mean time to positivity for all positives combined (PKDL + leprosy) was 13:25 minutes. The specificity of the m-LAMP method was 100% (95% CI: 94.0–100%) with 20 controls and 40 samples of other disease (leprosy/PKDL).

For the 40 leprosy samples, the amplification time ranged from 1:23 minutes to 31:22 minutes, with melting temperature ranging from 89.8°C to 90.58°C and mean Tm as 90.25 ± 0.20°C for *M. leprae*. The amplification time for the 40 PKDL samples varied from 2:23 minutes to 12:30 minutes, with a Tm for *L. donovani* ranging from 88.65°C to 89.32°C (mean Tm = 88.88 ± 0.17°C).

The case reporting with coinfection was categorized as macular PKDL and MB leprosy with neuritic involvement based on the clinical manifestation. The histopathology report showed focal perivascular collection of histiocytes and few plasma cells. Superficial peroneal nerve infiltration with foamy histiocytes and lymphocytes was observed, and a bacteriological index of 4+ was given based on Fite Faraco staining. The patient blood serum tested positive in the rK39 strip test, indicating the presence of *L. donovani*. We applied RLEP-based and kDNA-based QPCR to pretreated slit aspirate samples for the detection and quantification of *M. leprae* and *L. donovani*. The *M. leprae* count was found to be 254 bacteria/μL, whereas kDNA-based QPCR showed *L. donovani* load as 79 parasites/μL. Our m-LAMP assay successfully detected *M. leprae* and *L. donovani* in the slit aspirate sample in 13:05 minutes with distinct Tm values of 90.50°C and 88.97°C, respectively (Table 3, Figure 2B).

DISCUSSION

Both leprosy and PKDL are co-endemic tropical diseases with similar clinical manifestations that often lead to misdiagnosis, and thus, differential diagnosis is critical for controlling transmission of these diseases.[7,8,25]

In this study, a simple and rapid assay for differential detection of *M. leprae* and *L. donovani* based on the real-time multiplex LAMP technique was developed. Multiplex loop-mediated isothermal amplification assays have been used for detection and differentiation of various viruses and their subtypes and different bacterial diseases.[26–28] The portable real-time fluorometer used in this study incorporates the melting curve analysis, which allows the detection and discrimination of amplified products from a mixture by their different Tm values. Also, primer concentration adjustments were carried out to achieve the optimal amplification time with highest fluorescence reading and to minimize preferential amplification of one target gene over another. This primer concentration optimization along with the use of commercially available isothermal master mix containing an improved polymerase and a real-time fluorometer decreased the amplification times by 5-fold for both leprosy and PKDL in comparison with conventional LAMP (data not shown). Therefore, our m-LAMP assay represents a significant improvement over presently available assays for detecting both *M. leprae* and *L. donovani*. The high specificity of primers used in this study has been explored previously, and there were no false positives in our m-LAMP assay showing 100% specificity. The detection limit for simultaneous

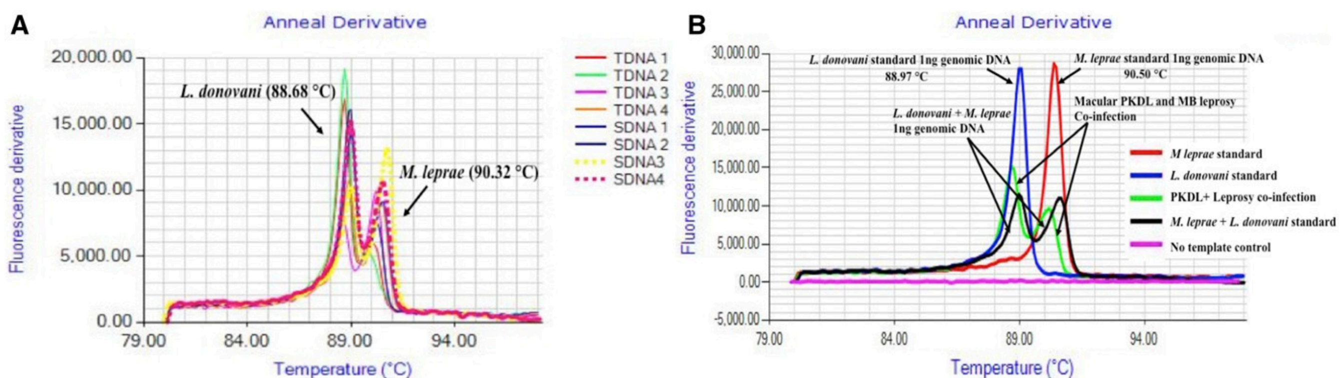

FIGURE 2. Diagnostic sensitivity of multiplex loop-mediated isothermal amplification assay for simultaneous detection of *Mycobacterium leprae* and *Leishmania donovani*. (**A**) In tissue (TDNA) and slit aspirate samples (SDNA) of leprosy and post–kala-azar dermal leishmaniasis (PKDL) patients combined. (**B**) In slit aspirate sample of patients coinfected with leprosy and PKDL, the melting curve shows two melting peaks (88.97°C for *L. donovani* and 90.50°C for *M. leprae*) distinguishing *L. donovani* and *M. leprae* amplification products. This figure appears in color at www.ajtmh.org.

TABLE 3

Amplification time and annealing temperatures of *Mycobacterium leprae* and *Leishmania donovani* in the slit aspirate sample of the patient coinfected with leprosy and post–kala-azar dermal leishmaniasis

Sample	Amplification (mm:ss)	Anneal (°C)
L. donovani 1 ng + *M. leprae* 1 ng	09:51	88.97°C; 90.49°C
L. donovani 1 ng	7:32	89.04°C
M. leprae 1 ng	9:38	90.50°C
Coinfected slit aspirate sample	13:05	88.97°C; 90.50°C

detection of *M. leprae* and *L. donovani* was found to be 100 fg/μL and 1 fg/μL, respectively, which was identical to that of individual LAMP. As shown in the data, the two target sequences were simultaneously amplified with mean Tm values for *M. leprae* and *L. donovani* in mixed reaction as 90.32 ± 0.22°C and 88.68 ± 0.05°C, respectively, and were, thus, clearly distinguishable. In addition, the sensitivity of the m-LAMP assay is comparable with that of QPCR and other PCR-based molecular methods.[10,29,30] Consequently, it is beneficial to have simultaneous detection of two or more diseases in a single reaction that can save considerable time, cost, and effort. Such an approach is particularly advantageous in detecting diseases with similar clinical manifestations to overcome the incidence of misdiagnosis.

The diagnostic sensitivity of the m-LAMP assay was determined by testing 80 QPCR-confirmed leprosy and PKDL samples. All 40 PKDL and 40 leprosy samples were positive in their individual LAMP assays, and the combined sensitivity of the m-LAMP assay for detecting PKDL and leprosy was 100% and 95%, respectively. In our study, amplification bias of *L. donovani* over *M. leprae* was observed in spite of testing different primer concentrations. This could be the reason for missing two slit aspirate leprosy samples in m-LAMP. Liu et al.[31] have reported a similar pattern with preferential amplification of the *Salmonella* target sequence over that of *V. parahaemolyticus*. For the present study, along with tissue samples, we used slit aspirates to minimize the invasive procedure of sample collection. The detection sensitivity of slit aspirates (95%) by m-LAMP was comparable with that of the tissue samples (100%). Slit aspirate sampling has advantages of ease of collection, storage, and transportation and most importantly can be effective in case–contact studies.

There are very few studies focusing on development of m-LAMP for simultaneous detection of distinct disease-causing organisms.[26,31,32] Our study is the first attempt to develop an m-LAMP assay for clinical application to co-endemic diseases PKDL and leprosy. Furthermore, a rare incidence of a confirmed case, coinfected with macular PKDL and leprosy, was accurately diagnosed by the m-LAMP assay using a slit aspirate sample of the patient and both *M. leprae* and *L. donovani* was detected. This finding strongly supports the potential of m-LAMP assay for leprosy and PKDL diagnosis, especially in endemic areas.

In conclusion, our data provided a foundation for differential and quick diagnosis of both leprosy and PKDL. Its utility can be evaluated in the field conditions to check the potential of the m-LAMP assay as a diagnostic tool. Nevertheless, because the endemic areas for leprosy and PKDL largely overlap, the assay developed in this study may improve the effectiveness and efficiency of the diagnosis and control of disease transmission by reducing the cost and time of diagnosis.

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Note: Supplemental table appears at www.ajtmh.org.

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Authors' addresses: Shweta Joshi, Keerti K. Dixit, Vanila Sharma, Ruchi Singh, and Poonam Salotra, National Institute of Pathology, Molecular Parasitology Laboratory, New Delhi, India, E-mails: shweta26jan83@gmail.com, keerti.dixit@gmail.com, vanilasharma81@gmail.com, ruchisp@gmail.com, and poonamsalotra@hotmail.com. V. Ramesh, Dermatology and Venereology, Vardhman Mahavir Medical College and Safdarjung Hospital, New Delhi, India, E-mail: weramesh@gmail.com.

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