
Original

Detection of Fungi from an Indoor Environment using Loop-mediated Isothermal Amplification (LAMP) Method

TAKAKO NAKAYAMA¹, TAKASHI YAMAZAKI^{2,3*}, AYAKA YO², KAZUYA TONE²,
MOHAMED MAHDI ALSHAHNI³, RYUICHI FUJISAKI⁴, AND KOICHI MAKIMURA^{1,2,3}

¹Division of Clinical Laboratory Medicine, Graduate School of Medical Care and Technology,

²Laboratory of Space and Environmental Medicine, Graduate School of Medicine,

³General Medical Education Research Center, and

⁴Emergency Room (ER), Department of Emergency -Medicine, Faculty of Medicine, Teikyo University,
2-11-1 Kaga, Itabashi Tokyo 173-8605, Japan

Received 25 November, 2015/Accepted 2 December, 2016

Loop-mediated isothermal amplification (LAMP) is a useful DNA detection method with high specificity and sensitivity. The LAMP reaction is carried out within a short time at a constant temperature without the need for thermal cycling. We developed a LAMP primer set for detecting a wide range of fungi by aligning the sequences of the large subunit ribosomal RNA gene of *Candida albicans* (Ascomycota), *Cryptococcus neoformans* (Basidiomycota), and *Mucor racemosus* (Mucorales). The threshold of *C. albicans* rDNA as template with our LAMP primer set was in the range of 10-100 copies per a reaction. In this study, we evaluated the correlation between colony forming units (CFU) and LAMP detection rate using the LAMP method for environmental fungi. The LAMP method should be a useful means of detecting fungi in indoor environments, disaster areas, or even in confined manned spacecraft to prevent allergies or infections caused by fungi.

Key words : Environmental fungi / Detection of fungi / Loop-mediated isothermal amplification (LAMP)/ Procedure for Ultra Rapid Extraction (PURE).

INTRODUCTION

Microbes in the environment have a potential impact on human health. In particular, excessive fungal growth may cause allergies, mycotoxicoses or serious mycoses (Fung and Hughson, 2008). Levels of fungal spores in the ambient air have been correlated with the number of asthma patient visiting to the emergency department (Dales et al., 2000). Several mycotoxins cause health problems among people living or working in damp and moldy environments (Miller and McMullin, 2014). Some environmental fungi are also causative agents of opportunistic infections (Ariza-Heredia and Kontoyiannis, 2014).

Culture-based techniques are still used to detect fungal contamination; however, this approach is time

consuming, presents a biohazard risk, and the ability to support the growth of fungal strains depends on the medium. Thus, quantitative polymerase chain reaction (PCR) methods for detecting and identifying fungi have been developed and widely utilized (Vesper et al., 2007).

Recently, the loop-mediated isothermal amplification (LAMP) method (Eiken Chemical Co., Ltd., Tochigi, Japan) has been used for detecting microbes, not only for medical purpose but also for environmental control. LAMP is a DNA amplification and detection method that involves an enzymatic reaction for DNA elongation at a constant temperature of 60-65°C for 1-2 h. Detection of pathogenic microbes, bacteria (Iwamoto et al., 2003; Lu et al., 2011; Soleimani et al., 2013; Ushijima et al., 2014), viruses (Kurosaki et al., 2016; Wheeler et al., 2016), and parasites (Gallas-Lindemann et al., 2016; Polley et al., 2013) has been reported based on the LAMP method, and some fungal species-specific

*Corresponding author. Tel: +81-3-3964-2140, Fax: +81-3-3964-8413, E-mail : yamazaki(a)med.teikyo-u.ac.jp

methods are available (Endo et al., 2004; Kawano et al., 2015; Ohori et al., 2006; Uemura et al., 2008; Yo et al., 2016).

In this study, we developed a LAMP method using a primer set for detecting a wide range of fungi from environmental specimens to evaluate the fungal burden, which should help to prevent allergies or infections caused by fungi.

MATERIALS AND METHODS

Oligonucleotide primers for the LAMP method

Fungal nucleotide sequences were obtained from the NCBI (National Center for Biotechnology Information) database (<http://www.ncbi.nlm.nih.gov>), and then multiple alignments of fungal DNA sequences were made using Genetyx Mac Ver. 12 software (Genetyx Co. Ltd., Tokyo, Japan). The LAMP primer set (F3, B3, FIP, and BIP; TABLE 1), designed to detect fungal species, but not human DNA, was homologous to sequences of the D1/D2 regions of the large subunit ribosomal RNA genes (LSU rDNA) of *Candida albicans* (DDBJ/EMBL/GenBank accession no. X70659) as a member of the Ascomycota, *Cryptococcus neoformans* (L14068) as a member of the Basidiomycota, and *Mucor racemosus* (M26190) as a member of the Mucorales, and *Homo sapiens* (L20636).

To assess the minimum detection limit with these LAMP primers, the plasmids carrying *C. albicans* DNA (plasmid DNA) were constructed as the template DNA for the LAMP method. The *C. albicans* TIMM1789 D1/D2 region of LSU rDNA was amplified with the primer pair 28SF1 (5'-AAGCATATCAATAAGCGGAGG-3') and 635 (5'-GGTCCGTGTTTCAAGACGG-3') (Sugita et al., 2003), and the PCR products were purified with a QIAquick PCR Purification Kit (Qiagen, Venlo, The Netherlands). The products were cloned with a TA Cloning Kit with the pCR2.1 vector (Life Technologies, Tokyo, Japan), and plasmids carrying *C. albicans* DNA were purified with a PureYield Plasmid Miniprep System (Promega, Madison, WI). Plasmid DNAs at 1×10^0 to 1×10^4 copies/ μ L was used in a LAMP reaction at 60°C for 90 min using the RT-160C real-time turbidity measurement system (Eiken Chemical Co., Ltd.). The LAMP reaction was terminated by denaturing the DNA poly-

merase at 80°C for 5 min. The reaction mixture (25 μ L) contained 80 pmol each of inner primer pair (FIP and BIP) and 10 pmol each of outer primer pair (F3 and B3).

To assess the specificity of these LAMP primers for the fungal species, standard fungal strains of 36 species and 23 genera and 2 nonfungal species were used (TABLE 2). Potato dextrose agar (PDA) plates (Kanto Chemical Co., Inc., Tokyo, Japan) supplemented with 100 μ g/mL chloramphenicol were used to culture fungi at 28°C for up to 2 weeks. The total DNA was extracted from fungal cells grown on PDA plates using the NucliSENS miniMag system (bioMérieux, Lyon, France) according to the manufacturer's instructions or by phenol-chloroform extraction (Makimura et al., 2000). Aliquots of 1 ng of each genomic DNA were added to individual LAMP assays as template.

Comparison of DNA extraction methods

Specimens were collected from four areas of our laboratory (lab bench, side of a freezer as a vertical surface, freezer top as a horizontal surface, and air conditioner outlet, 25 cm² each) using Large Alpha Swabs (Texwipe, Kernersville, NC USA) moistened with distilled water (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan). Each of these swabs was washed with an aqueous solution of 1 mL 0.05% Tween 80 to release and retrieve fungal cells and spores. Aliquots of 20 and 200 μ L were used for cultivation and another aliquot of 200 μ L was used for DNA extraction. After freeze-thawing three times, DNA was extracted from the specimen solution by a NucliSENS miniMag system or Procedure for Ultra Rapid Extraction (PURE) kit (Eiken Chemical Co., Ltd.) (Kawano et al., 2015; Mitarai et al., 2011) according to the manufacturer's instructions. The process required 1 h for the NucliSENS MiniMag system and 15 min for the PURE kit. Extracted DNA was stored at -80°C until use.

Detection of fungi in the indoor environment

Swab specimens were collected from total 60 points of four areas in our laboratory (100 cm² each) according to the method described above. Each aliquot of 200 μ L of swab specimens was tested by the LAMP method, as described above. On the other hand, aliquots of 20

TABLE 1. Sequences of the LAMP primers used in this study.

Primer	Sequence (5'→3')
F3	GAGGGTGAGAATCCCGT
B3	CTTCCCTTTCAACAATTTAC
FIP	CCAATATTAGCTTTAGATGGAATTGAGTCGAGTTGTTTGGA
BIP	AGACCGATAGCGAACAAGTACTACTTTTCACTCTCTTTCAAAGT

TABLE 2. Fungal species and strains used in the LAMP assay to determine specificity.

Phylum	Order	Species	Strain	Amplification by LAMP primers ⁱ
Ascomycota	Onygenales	<i>Trichophyton rubrum</i>	TIMM1824 ^a	+
		<i>Trichophyton tonsurans</i>	NBRC5928 ^b	+
		<i>Trichophyton mentagrophytes</i> (<i>Arthroderma vanbreuseghemii</i>)	TIMM2789	+
		<i>Trichophyton mentagrophytes</i> (<i>Arthroderma benhamiae</i>)	SM103 ^c	+
		<i>Microsporum gypseum</i>	NBRC5948	+
		<i>Histoplasma capsulatum</i>	TIMM0713	+
	Eurotiales	<i>Aspergillus fumigatus</i>	TIMM2920	+
		<i>Aspergillus flavus</i>	JCM2061 ^d	+
		<i>Aspergillus niger</i>	TIMM2932	+
		<i>Aspergillus oryzae</i>	TIMM0117	+
		<i>Aspergillus versicolor</i>	TIMM1290	+
		<i>Aspergillus japonicus</i>	TIMM2910	+
		<i>Paecilomyces variotii</i>	NBRC4855	+
		<i>Penicillium expansum</i>	TIMM1293	+
	Chaetothyriales	<i>Exophiala jeanselmei</i>	TSY-0396 ^e	+
	Helotiales	<i>Scytalidium lignicola</i>	NBRC104988	+
	Microascales	<i>Pseudallescheria boydii</i>	TIMM3071	+
		<i>Scopulariopsis brevicaulis</i>	NBRC4843	+
		<i>Scopulariopsis brumptii</i>	NBRC6441	+
	Hypocreales	<i>Fusarium solani</i>	TSY-0403	+
		<i>Fusarium oxysporum</i>	TSY-0351	+
		<i>Acremonium curvulum</i>	NBRC32242	+
	Sordariales	<i>Chaetomium globosum</i>	TSY-0369	+
	Pleosporales	<i>Alternaria alternata</i>	TSY-0854	+
	Capnodiales	<i>Cladosporium carionii</i>	TIMM3048	+
	Saccharomycetales	<i>Candida albicans</i>	TIMM1789	+
		<i>Candida glabrata</i>	ATCC90030 ^f	+
		<i>Candida krusei</i>	ATCC6258	+
		<i>Candida tropicalis</i>	TIMM0313	+
Basidiomycota	Sporidiobolales	<i>Rhodotorula minuta</i>	TIMM6222	+
	Malasseziales	<i>Malassezia restricta</i>	CBS7877 ^g	+
	Tremellales	<i>Cryptococcus neoformans</i>	ATCC90113	+
		<i>Trichosporon asahii</i>	CBS2479	+
Incertae sedis	Mucorales	<i>Mucor circinelloides</i>	TIMM3177	+
		<i>Cunninghamella bertholletiae</i>	TIMM3391	+
		<i>Rhizopus oryzae</i>	TIMM1326	+
Nonfungal species				
<i>Mycobacterium bovis</i>			BCG Tokyo	—
<i>Homo sapiens</i>			— ^h	—

^aSource, The Teikyo University Institute of Medical Mycology (Japan)^bSource, NITE Biological Resource Center (Japan)^cSource, Department of Dermatology, Shiga University of Medical Science (Japan)^dSource, Japan Collection of Microorganisms (Japan)^eSource, National Institute of Health Sciences (Japan)^fSource, American Type Culture Collection (Manassas, VA)^gSource, The Convention on Biological Diversity (The Netherlands)^hThe commercial human DNA preparation G147A (Promega Corp., Madison, WI)ⁱ+, amplification observed after 90 min of incubation; —, amplification not observed after 90 min of incubation

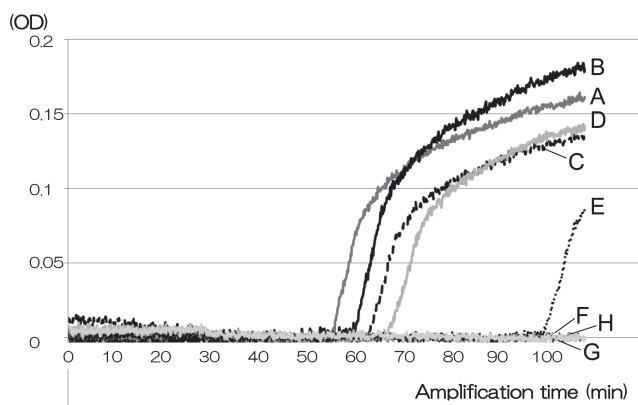


FIG. 1. Sensitivity of the LAMP primers for *C. albicans* for different numbers of plasmid DNA.

A: Positive control, the concentration of template DNAs of B: 1×10^4 , C: 1×10^3 , D: 1×10^2 , E: 1×10^1 , F: 1×10^0 , G: Negative control (1), H: Negative control (2)

μL or 200 μL from swabs were spread on PDA plates and incubated at 28°C for up to 7 weeks. All colonies growing on PDA plates were counted and identified by microscopic observation and/or LSU rDNA sequencing. DNA sequencing was performed as follows, fungal cells were suspended in lysis buffer (20 mM Tris-HCl, pH 8.0, 25 mM EDTA, 1% w/v SDS, 250 mM NaCl) and ground with a BioMasher disposable homogenizer (Nippi, Ibaraki, Japan). Lysates were centrifuged at 15,000g for 10 min, the supernatants were isolated, and DNA was extracted using phenol-chloroform, then purified by ethanol precipitation. The LSU rDNA of each fungal colony was amplified with the primer pair 28SF1 and 635. PCR products were purified and sequenced using an Applied Biosystems 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA USA). DNA sequences were identified using the BLAST algorithm (Basic Local Alignment Search Tool at NCBI).

RESULTS

Minimum detection limit and specificity of LAMP primers

To examine the sensitivity of the primer sets, the LAMP reaction was first performed with plasmid DNA as the template. As shown in Fig.1, amplification curves for the LAMP reaction were examined using 10^0 , 10^1 , 10^2 , 10^3 , or 10^4 copies of template DNA per a reaction. The initial rise tended to occur earlier when the copy number of the template DNA was higher, although amplification of only 10 copies was extremely slow. These observations suggested that the time of initial rise was proportional to the concentration of the template DNA above a certain cut-off, and the threshold of this primer set was

TABLE 3. Number of colonies cultured on PDA plates from various areas of our laboratory.

Laboratory areas	CFU/200 μL
Lab bench	6.4
Freezer surface (vertical surface)	8.6
Freezer surface (horizontal surface)	16.4
Air conditioner outlet	53.4

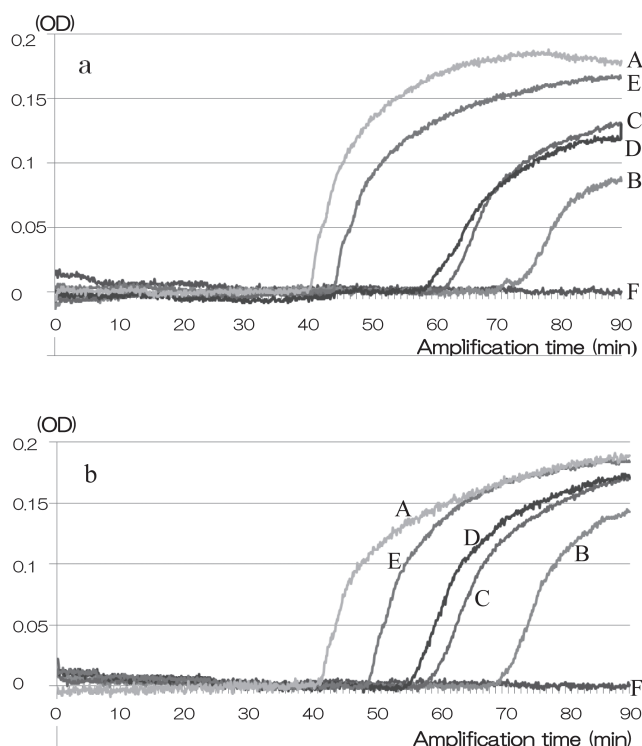


FIG. 2. Comparison of DNA extraction methods.

a: DNA extracted by NucliSENS miniMag system, b: DNA extracted by Plasmid Ultra Rapid Extraction (PURE) kit

A: Positive control, B: Lab bench, C: Freezer surface (vertical surface), D: Freezer surface (horizontal surface), E: Air conditioner outlet, F: Negative control

concluded to lie within the range of 10 to 10^2 copies of *C. albicans* plasmid DNA.

To assess specificity, genomic DNAs of various fungal species (TABLE 2) were used as template in the LAMP reaction. The results suggested that this primer set can be used for detection of a wide range of fungal species, whereas amplification of DNA from nonfungal species did not occur.

Comparison of DNA extraction methods

The number of colonies forming on a PDA plate from the swab collected from each area of the laboratory is shown in TABLE 3, and the amplification curves of the

TABLE 4. Correlation of colony number on plates and positive rate for LAMP method.

	CFU ^b =0	10>CFU≥1	CFU≥10	Total number of swab specimens
Number of swab specimens	32	17	11	60
Number of swab specimens with positive reaction by LAMP assays	2	2	7	11
Positive rate (%) ^a	6.3	11.8	63.6	18.3

^aPositive rate (%) = Number of positive LAMP assays / Number of specimens × 100, indicating the LAMP positive rate in each CFU range.

^bCFU / 200 µL counted on the PDA plate.

LAMP reaction using the DNA from two extraction methods are shown in Fig.2. The time of initial rise in the LAMP reaction showed the same tendency in both of DNAs from two extraction methods, and was correlated with the number of colonies. This result suggested that the more rapid DNA extraction method using the PURE extraction kit is more useful than the NucliSENS miniMag system, for extracting DNA from fungi in the environment including indoor living environments.

Detection of fungi in the environment using the LAMP method

The comparison of colony number to LAMP results with swab specimens collected from each area of the laboratory is shown in TABLE 4. The results in this study showed that fungal colonies were observed on PDA plates in 46.7% (28 of 60) of overall specimens, but the LAMP method positively reacted in only 18.3% (11 of 60). Most of the specimens with few colonies tended to show a negative reaction in the LAMP method. The positive rate from the LAMP method seemed to increase with the increasing the number of colonies formed on PDA plates, although some swab specimens showed a positive reaction in the LAMP method despite an absence of colonies on PDA plates.

The fungi detected from swab specimens obtained from 60 points in four areas in our laboratory are shown in TABLE 5. It is noteworthy that *Cladosporium* spp. and *Aspergillus* spp. were detected from 63.6% (7 of 11) of all swab specimens with positive reactions by the LAMP method. The rates of appearance of these two genera were higher than the others, and showed similarly high detection rates, 60.7% (17 of 28) and 50.0% (14 of 28), by the culture-based method. The detection rates by this LAMP method were proportional to the rates by culture-based method, regardless of the fungal genus.

DISCUSSION

In the swab specimens with CFU ≥ 10 on PDA plates, 63.6% were positive by the LAMP method (TABLE 4); however, the specimens with CFU < 10 showed a decidedly lower reaction rate (11.8%). These results indicated that the sensitivity of this LAMP method is proportional to CFU and the concentration of template DNA, and is thus more effective when fungi are present in larger numbers. Although the few specimens with CFU = 0 showed a positive reaction in the LAMP method (6.3%), there is a possibility that a positive reaction was derived from DNA of nonviable cells because the LAMP can detect the presence of DNA fragments containing the target sequence.

The detection rate of the LAMP method was lower than that of the culture method; however, the LAMP method using our primer set has the advantage of providing results within 1-2 h, which is considerably faster than the periods required to detect and identify fungi by the culture-based method. The PURE DNA purification method was used for fungal DNA extraction with minor modifications. Generally, centrifuges and special reagents are necessary to extract and purify fungal DNA for LAMP (Ghosh, et al., 2015; Uemura et al., 2008). However, the PURE extraction kit used in this study is handled easily and does not require any special equipment other than a freezer for freeze-thawing. This method is advantageous because it is simpler than many conventional fungal DNA extraction techniques. Thus, it can reduce not only the time required, but also the number of steps in the DNA extraction process, leading to a reduction in the risk of cross-contamination. Furthermore, the LAMP reaction time would be expected to be shortened by adding two oligonucleotides containing complementary sequences to the single-stranded loop region of LAMP products, the so-called loop primers (Nagamine et al., 2002).

Cladosporium spp. and *Aspergillus* spp. were the dominant fungal genera isolated in our laboratory using

TABLE 5. The positive rates of swab specimens and frequency of each fungal genus by cultures and the LAMP method

Fungal genus	Culture-based method (CFU $\geq$ 1)		LAMP method	
	Number of positive specimens	Rate (%) ^c	Number of positive specimens	Rate (%) ^d
Number of swab specimens with positive reaction		28	11	
<i>Cladosporium</i>	17	60.7	7	63.6
<i>Aspergillus</i>	14	50.0	7	63.6
<i>Rhodotorula</i>	9	32.1	3	27.3
<i>Chaetothyriales</i>	3	10.7	3	27.3
<i>Penicillium</i>	3	10.7	2	18.2
<i>Chaetomium</i>	3	10.7	1	9.1
<i>Arthrimum</i>	2	7.1	1	9.1
<i>Paraconiothyrium</i>	1	3.6	1	9.1
<i>Didymella</i>	1	3.6	1	9.1
<i>Sporidesmium</i>	1	3.6	1	9.1
<i>Microstoma</i>	1	3.6	1	9.1
<i>Parastagonaspora</i>	1	3.6	1	9.1
<i>Epicoccum</i>	1	3.6	1	9.1
Yeast ^a	1	3.6	1	9.1
<i>Pleurotus</i>	2	7.1	0	0.0
Elsinoaceae ^b	1	3.6	0	0.0
<i>Cunninghamella</i>	1	3.6	0	0.0
<i>Sporisorium</i>	1	3.6	0	0.0
<i>Alternaria</i>	1	3.6	0	0.0
<i>Trametes</i>	1	3.6	0	0.0
<i>Lophiostoma</i>	1	3.6	0	0.0
<i>Paecilomyces</i>	1	3.6	0	0.0
<i>Tilletiopsis</i>	1	3.6	0	0.0
<i>Nectriaceae</i>	1	3.6	0	0.0
<i>Bjerkandera</i>	1	3.6	0	0.0
<i>Eutypa</i>	1	3.6	0	0.0
<i>Valsa</i>	1	3.6	0	0.0

^aYeast could not be classified further than *Rhodotorula*.

^bElsinoaceae is a family, not genus. It could not be further classification.

^cRate (%) = Number of swab specimens with detection of each fungal genus by the culture-based method / number of positive specimens by the culture-based method (CFU $\geq$ 1) $\times$ 100

^dRate (%) = Number of swab specimens with detection of each fungal genus by the LAMP method / number of positive specimens by the LAMP method $\times$ 100

the culture method, and these two genera were detected similarly in the swab specimens with LAMP-positive reactions. The frequency of appearance of fungal genera by the LAMP method was almost the same as by the culture-based method, although it is necessary to scrutinize the correlation between the CFU and the detection rate of each genus by the LAMP method.

Using a combination of PURE DNA extraction and

the LAMP method is expected to be useful in environments with limited laboratory resources that require continuous microbial monitoring, because it is simple to perform and yields rapid results, allowing diagnosis of the source of fungal diseases and confirmation of microbial control. Such a method will allow rapid confirmation of the presence of fungi in various resource-limited environments, such as areas affected by disas-

ters or manned spacecraft (Nukiwa, 2012; Satoh et al., 2011 and 2016; Shirakawa et al., 2015).

In conclusion, we have developed a LAMP-based evaluation method for detecting environmental fungi and presented an example of its practical use. Although our data were limited, further validation will demonstrate the applicability of the LAMP method. Our LAMP method is a promising candidate for a simple molecular evaluation system for prevention and diagnostic of fungal hazards in indoor environment, disaster areas, or even in confined manned spacecraft to prevent allergies or infections caused by fungi.

ACKNOWLEDGMENTS

The authors thank Ms. Yoshiko Umeda for her technical help and advice. The authors declare no conflict of interest. This work was supported in part by MEXT Grant-in-Aid for Scientific Research on Innovative Areas, Number 15H05946.

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