# Chapter 18
# Applications of Loop-Mediated Isothermal Amplificaton Methods (LAMP) for Identification and Diagnosis of Mycotic Diseases: Paracoccidioidomycosis and *Ochroconis gallopava* infection

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**Abstract** Loop-mediated isothermal amplification (LAMP) methods are now useful for the detection of a specific gene in infectious diseases, genetic diseases, and/or genetic disorders in the large number of medical fields, and it was recently introduced to fungal investigation. It is characterized by the use of four different primers specifically designed to recognize six distinct regions of the target gene, and the reaction process proceeds at a constant temperature using strand displacement reaction. Quickness and simplicity is the advantage of the method. Amplification and detection of gene can be completed in a single step, by incubating the mixture of samples, primers, DNA polymerase with strand displacement activity and substrates at a constant temperature. The method was applied to two fungal infections; paracoccidioidomycosis (PCM), a deep mycosis caused by *Paracoccidioides brasiliensis* and *Ochroconis gallopava* infection. For PCM a combination of F3, B3, FIP, and BIP primers designed from the partial sequence of *P. brasiliensis gp43* gene was used. The PCR products amplified by the primer set; F3 and B3 showed species specificity for *P. brasiliensis* and the detection limit of the PCR was 100 fg of fungal genomic DNA. The specific DNA banding pattern of *P. brasiliensis* was detected in the clinical and nine-banded armadillo derived isolates, paraffin-embedded tissue sample or sputum from PCM patient. LAMP method was used also for the identification of *O. gallopava* by using species-specific primer sets based on the D1/D2 domain of the LSU rDNA sequence. The method successfully detected the gene from both fungal DNA derived from brains and spleens of

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experimentally-infected mice with *O. gallopava* and environmental isolates. In conclusion, LAMP method for PCM and *O. gallopava* seemed to be useful for identification, diagnosis or retrospective study with advantage in the quickness and simplicity procedure, but require strictly-controlled environments.

## 18.1 Introduction

The gold standard for diagnosis of fungal infections is detection, isolation and identification of the patogenic fungi from clinical specimen; such as skin scrapings, hair and nail, mouth and vaginal swabs, blood, cerebrospinal fluid, urine, sputa and respiratory tract secretion, pus, ocular specimen, and organ biopsy. However, there are many complicated problems in clinical laboratories.

First of all, the most important procedure is avoiding laboratory infections caused by highly pathogenic fungal species, such as *Coccidioides immitis*, *C. posadasii*, *Histoplasma capsulatum*, *Blastomyces dermatitidis*, *Paracoccidioides brasiliensis* and *Penicillium marneffei*. In general, highly pathogenic fungal species are not recommended to isolate clinical laboratories in general (Larone 1995). Furthermore, a fungal infection, caused by *Ochroconis gallopava,* which requires to be differentiated from highly pathogenic bird flu or SARS (Ohori et al. 2006), is also recommended to diagnose without culturing to avoid the viral laboratorial infections.

The lower rate of isolation of the causative agents seems to be caused by short incubation periods for the isolation of fungi from clinical material. Visualization of fungal sprouts from clinical materials takes a longer time than those of bacteria. It takes at least 48 h in pathogenic yeasts, 4–7 days in common pathogenic filamentous fungi; dermatophytes, *Aspergillus* spp., zygomycetes, and others, almost 10–14 days in dematiaceous fungi, and 3 weeks or more in particular fungal species, especially *P. brasiliensis,* of course, it should not try to isolate in general laboratories. But most laboratories could not keep incubating more than 1 week. Therefore, the isolation rate of filamentous fungal pathogens seems to be very low compared to pathogenic yeasts.

Followed by difficulties in isolations of fungal pathogens, there is a serious problem with identification. Expert skills for identification based on morphological and physiological characteristics are strongly required. The procedures are highly time-consuming to prepare photogenic samples and to evaluate special morphologiclal and physiological characteristics (Ohori et al. 2006). Furthermore, clinical isolates sometimes lacked characteristic appearances such as textures and colors of colonies, conidiogenesis, mating and physiological abilities (Uno et al. 2001).

Diagnosis for fungal infections is able to be confirmed on the basis of combinations of clinical findings, diagnositic imagings, serological tests, immunological

tests, cytological and histopathological findings without the fungal isolate (Ishikawa et al. 2008).

Detections of 1.3-beta-D-glucan, galactomannan, and D-arabinitol from sera are faster than other methods, and are also useful for diagnosis of some fungal infections (Christensson et al. 1999; Kelaher 2006), however, it is impossible to estimate the species of the causative agent.

Isolation and identification, and detection of a species-specific gene by molecular biological methods from clinical materials are able to confirm the causative agent in the species level (Borman et al. 2008).

Following the developments of molecular biological techniques in these two decades, molecular biological data for identification of fungal species based on ribosomal DNA (rDNA) sequences became common, such as for *P. brasiliensis* identification (Motoyama et al. 2000). Although internal transcribed spacer 1 region of rDNA sequence (ITS 1 rDNA) is treated as a barcode gene for identification and taxonomical classification of fungi (Druzhinina 2005), there were some difficulty to confirm the fungal species based on the gene sequences. Therefore, selection of species-specific genes, beside the ITS 1 rDNA may add value to targets.

Detections of species-specific genes derived from causative agents in clinical materials by polymerase chain reactions (PCR) have been reported in many mycotic diseases (Reiss et al. 2000; Balajee et al. 2007). But, it still takes at least several hours to obtain the results and sometimes should be requested to confirm the sequences of the amplified genes. Therefore, rapid and accurate diagnostic methods based on molecular biolgical techniques have been waited for.

Notomi et al. in 2000 reported a new method, the so called loop-mediated isothermal amplification (LAMP) method to detect specific gene from a DNA virus within a few hours. The method has been applied in the field of microbiology for detection and identification of *Mycobacterium* sp. (Iwamoto et al. 2003), *hepatitis B* virus (Nagamine and Watanabe 2001; Nagamine et al. 2002), highly pathogenic bird flu (Imai et al. 2007) and many other pathogens reaching to more than 200 reports up to the end of November 2008.

On the other hand, the technique has not been successful in applying fungal infections. Personal communications suggested that the LAMP methods are useful for opportunistic fungal infection in the early times; however, our opinion is that the LAMP methods for *Candida* spp., *Aspergillus* spp., and other opportunistic fungal species may not be reliable because of the difficulties to judge the real pathogen or environmental contaminant. We would like to suggest that the application of the LAMP methods to mycotic diseases should be limited to the highly pathogenic fungal species out of endemic areas, and/or to rare species, for example *Cryptococcus gattii* (Lucas et al. 2009), although a LAMP method for identification of *Candida* spp. was reported by Inácio et al (2008).

The present chapter describes the principle of LAMP method, detections of specific gene from *P. brasiliensis* categorized as one of the highly pathogenic fungi (Endo et al. 2004), and of *O. gallopava* required to be differentiated from highly pathogenic bird flu or SARS (Ohori et al. 2006).

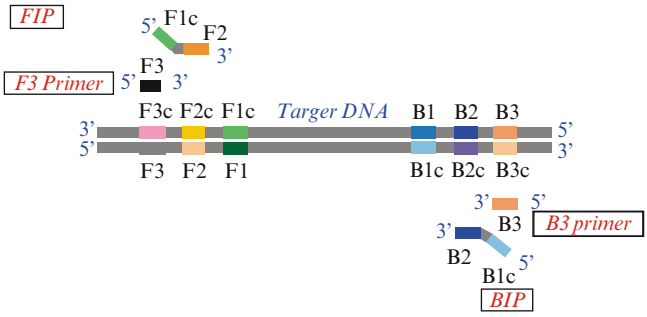


**Fig. 18.1** Design 4 types of primers based on the following six distinct regions of the target gene: the F3c, F2c and F1c regions at the 3′ side and the B1, B2 and B3 regions at the 5′ side. FIP: Forward Inner Primer (FIP) consists of the F2 region (at the 3′ end) that is complementary to the F2c region, and the same sequence as the F1c region at the 5′ end. F3 Primer: Forward Outer Primer consists of the F3 region that is complementary to the F3c region. BIP: Backward Inner Primer (BIP) consists of the B2 region (at the 3′ end) that is complementary to the B2c region, and the same sequence as the B1c region at the 5′ end. B3 Primer: Backward Outer Primer consists of the B3 region that is complementary to the B3c region (http://loopamp.eiken.co.jp/e/lamp/primer.html)

## 18.2 The Principle of LAMP Method

### *18.2.1 About LAMP Method (http://loopamp.eiken.co.jp/e/lamp/index.html)*

LAMP method which stands for LAMP is a simple, rapid, specific and cost-effective nucleic acid amplification method solely developed by Eiken Chemical Co., Ltd. It is characterized by the use of four different primers specifically designed to recognize six distinct regions on the target gene and the reaction process that proceeds at a constant temperature using strand displacement reaction (Fig. 18.1).

Amplification and detection of genes can be completed in a single step, by incubating the mixture of samples, primers, DNA polymerase with strand displacement activity and substrates at a constant temperature (about 65°C). It provides high amplification efficiency, with DNA being amplified $10^9$–$10^{10}$ times in 15–60 min. Because of its high specificity, the presence of amplified product can indicate the presence of the target gene.

### *18.2.2 Primers*

LAMP method uses four primer sets; F3, B3, FIP and BIP selected from six distinct regions of the target gene (http://loopamp.eiken.co.jp/e/lamp/primer.html). The most important primer set is F3 and B3. The primers should be selected from specific genes or gene sequences based on species specific PCR and confirmed after

testing with intra species diversity and a huge numbers of related pathogenic fungal species.

Therefore, enormous numbers of trials and errors are latent until the final primers are confirmed. Furthermore, the primers should completely differentiate the fungal genes from host ones.

Although, special software to design LAMP primers- PrimerExplore is available in the website (http://primerexplorer.jp/e/), it seemed to be useful as reference hints for base composition, GC contents, secondary structures and Tm value on designing primers based on our experience.

### *18.2.3 Basic Principle*

The target gene (DNA template as example) and the reagents are incubated at a constant temperature between 60–65°C. The reaction steps are available at the website (http://loopamp.eiken.co.jp/e/lamp/principle.html).

### *18.2.4 Cautions*

LAMP method is highly sensitive. We experienced many faults of contamination of the genes. Once contamination of the target gene occurs, all reactions become positive, even in a negative control using distilled water as a template. Therefore, extremely careful procedures are requested. Reagents, pipets, plastic pipet tips, safety cabinet, and hands. The samples should be handled separately from the reagents. The positive control for the target gene should be done separately.

This is one of the reasons why some fungal species common in normal human flora or in environments are not recommended to use the LAMP method. Selection of the target fungal species is also important. The fungal species should be rare in laboratorial environment.

## 18.3 Applications of LAMP Method for Identifications of *P. brasiliensis* and or/Diagnosis for Paracoccidioidomycosis (PCM)

### *18.3.1 Backgrounds for P. brasiliensis*

*P. brasiliensis* is considered to belong to the family Onygenaceae (Order Onygenales, Ascomycota), in the same group as *Blastomyces dermatitidis*, *Coccidioides immitis*, *Histoplasma capsulatum*, and *Lacazia loboi* (Bagagli et al. 2008). The

fungal species is treated as one of the highly pahogenic fungi categorized as biosafety level 3 as the same as *C. immitis*, *C. posadasii*, *H. capsulatum*, *B. dermatitidis* and *Penicillium marneffei* (Kamei et al. 2003) On the other hand, the identification and diagnosis of the above fungal infections with nonculture method seems to be very important to avoid laboratory infection (Kamei et al. 2003, Umeyama et al. 2006).

*P. brasiliensis* is the causative agent for paracoccidioidomycosis (PCM) endemic in Latin American countries. This fungus invades the lungs, lymph nodes, skin, mucosa, liver, spleen and various other organs of humans and dogs. In humans, the disease is characterized by two clinical forms: the acute or juvenile form (AF) and more frequently chronic or adult form (CF). The AF is prevalent in children and young people and presents a more severe and rapid clinical evolution with the involvement of multiple organs and adenomegaly, hepatosplenomegaly, digestive disorders, osteo-articular involvement and muco-cutaneous lesions. The CF occurs mainly in adult males and has multiple forms, ranging from benign and localized (unifocal) to severe and disseminated (multifocal) disease that involves skin, mucous membranes, pulmonary and lymph node manifestations (Restrepo 1985; Franco 1987; Kwon-Chung and Bennett 1992; Brummer et al. 1993; Ono et al. 2003; Ricci et al. 2004).

The probable natural habitat of *P. brasiliensis* is soil as saprophytic form. In fact, isolations from soil or soil related products, from the feces of both frugivorous bats (*Artibeus lituratus*) and a penguin (*Pygoscelis adeliae*) were reported. Interestingly, the natural reservoir of *P. brasiliensis* seems to be the nine-banded armadillo (*Dasypus novemcinctus*) because of repeated isolation of the fungal species from various endemic areas of paracoccidioidomycosis with high incidences showing, as the same genetic profiles as clinical isolates. Furthermore, detection of *P. brasiliensis* gene from the internal organs of wild animals that died in traffic accidents; guinea pig (*Cavia aperea*), porcupine (*Sphiggurus spinosus*), grison (*Gallictis vittata*) and raccoon (*Procyon cancrivoros*) suggested that the mycosis invades not only humans but also many mammal species, and is one of zoonotic mycosis (Bagagli et al. 2008).

The characteristics of *P. brasiliensis* is temperature-dependent dimorphism; a mycelial form at ambient temperature, and multiple budding yeast form in host tissue or at temperatures above 35–37°C in certain culture media (Restrepo 1985; Franco 1987; Franco et al. 1989; Kwon-Chung and Bennett 1992; Brummer et al. 1993). (Fig. 18.2).

The important characteristics of *P. brasiliensis* are a multiple-nuclei microorganism (Imai et al. 2000), and a haploid microorganism except for *gp43*, encoding the major antigen of *P. brasiliensis*; 43 kDa glycoprotein appeared as only one allele (Almeida et al. 2007). The mature or during maturation of yeast cells that have multiple nuclei, suggested that any *P. brasiliensis* gene may easily be amplified because of multiple copies at least. It suggested that selection of the target gene is free from sticking on ribosomal RNA genes having tandem repeats (Kobayashi 2006) from clinical materials.

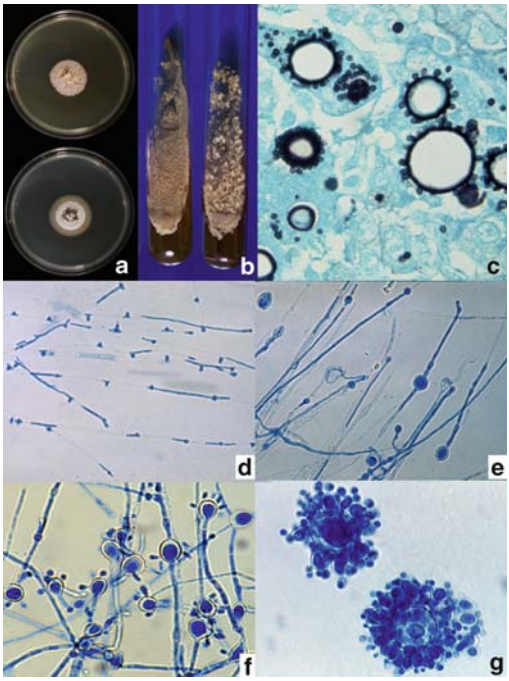


**Fig. 18.2** (**a**) Upper; colony of *P. brasiliensis* cultured on Sabouraud dextrose agar plate, lower; on potato dextrose agar plate at 25°C for 8 weeks, (**b**) cerebriform yeast-like colony on 1% dextrose added brain heart infusion agar slant cultured at 35°C for 7 days (left; nine-banded armadillo derived isolate, right; clinical isolate), (**c**) whip wheel like appearance in a infected lymphonode tissue (Dr. Nakajima Y, Matsushita Memorial Hospital, Osaka, Japan), (**d**) aleurioconidia cultured on potato dextrose agar at 25°C for 8 weeks, (**e**) clamydospores cultured on potato dextrose agar at 25°C for 8 weeks, (**f**) mycelial to yeast form conversion process cultured on potato dextrose agar at 25°C for 2 weeks and cultured at 35°C for 3 days, (**g**) multiple budding yeast cells consisted of big mother cells, daughter and grand daughter cells

Genetic data of *P. brasiliensis* have progressed in the twenty first century. More than 5,000 sequences of *P. brasiliensis* are released into the GenBank database (http://www.ncbi.nlm.nih.gov/sites/entrez).

Based on multiple gene analysis, *P. brasiliensis* was separated into three different phylogenetic species; S1 (species 1 from Brazil, Argentina, Paraguay, Peru and Venezuela), PS2 (phylogenetic species 2 from Brazil and Venezuela) and PS3 (phylogenetic species 3 from Colombia) (Matute et al. 2006a, b, 2007).

Whole genome sequences on three strains of *P. brasiliensis* (Pb01, Pb03 and Pb18) were released in the BROAD Institute (http://www.broad.mit.edu/annotation/genome/paracoccidioides_brasiliensis/MultiHome.html). According to Carrero et al. 2008, isolate Pb01 might be a new *Paracoccidioides* species because of its diversity of gene profiles compared to other *P. brasiliensis* isolates, and was named as *P. lutzii* (Teixeira et al. 2009).

Among various genes, the *gp43* is the most important gene because of its diagnostic value (Puccia et al. 2008). We have also been trying to detect *gp43* from paraffin embedded tissue samples and blood (Sano et al. 2001; Itano et al. 2002).

The gene encodes the major fungal antigen; 43 kDa glycoprotein, which is a dominant *P. brasiliensis* antigen, and has been used for serological test in endemic areas (Miura et al. 2001; Camargo 2008).

Approximately 300 sequences of *gp43* were released in the GenBank database at the end of August 2009. The gene homologies among the majority of *P. brasiliensis* isolates is more than 96% in, except for Pb01 and its related isolates identity (Teixeira et al. 2009, Takayama et al. 2009). According to Takayama et al., the LAMP band pattern of *P. lutzii* was different from those of *P. brasiliensis*.

## *18.3.2 LAMP Method for Identifications of P. brasiliensis*

### 18.3.2.1 *P. brasiliensis* Isolates and Reference Species

Twenty-two clinical and seven nine-banded armadillo (*Dasypus novemcinctus*) derived *P. brasiliensis* isolates were tested.

As an advanced notice, our method might limit to detect S1 phylogenetic type of *P. brasiliensis* since we have not tested the isolates belonging to PS2 and PS3 proposed by Matute et al. 2006a and b. Furthermore, there is an uncertainty to detect *gp43* in atypical isolate *P. brasiliensis* strain Pb01 and its related isolates (Carrero et al. 2008; Takayama et al. 2009), and has just been named as the new species *P. lutzii* (Teixeira et al. 2009).

Isolates of *Coccidioides immitis sensu lato* (IFM 50993, identified as *C. posadasii* based on multiple gene analysis by Sano et al. 2006), *Histoplasma capsulatum* (IFM 41329), *Blastomyces dermatitidis* (IFM 41316), *Sporothrix schenckii* (IFM 47068), *Penicillium marneffei* (IFM41708), *Candida albicans* (IFM 5740), and *Cryptococcus neoformans* (IFM 5830) were used as negative controls (Table 18.1).

### 18.3.2.2 Extraction of DNA

Isolates of *P. brasiliensis* were evaluated. Yeast-form cells harvested on 1.0% glucose added Difco™ brain heart infusion agar (Becton Dickinson Microbiology Systems, Sparks, MD, USA) slants at 35°C for 7 days were used. Approximately $5 \times 10^8$ yeast-form cells were suspended in distilled water (DW) and washed three times with DW, and homogenized in a 1.5 mL volume plastic homogenizer. DNA was extracted with the Gen Toru Kun for the yeast (Dr. GenTLE™ for yeast) kit (TAKARA BIO INC., Ohtsu, Shiga, Japan).

Isolates of *C. posadasii H. capsulatum*, *B. dermatitidis*, *S. schenckii*, *P. marneffei*, *Ca.albicans*, and *Cr. neoformans* were cultured on potato dextrose agar (Becton Dickinson Microbiology Systems) at 25°C for 7–60 days.

The fungal cells of *C. posadasii* were fixed with 70% ethanol overnight, and the DNA was extracted by the kit (Dr. GenTLE™ for yeast, TAKARA BIO INC.). The final concentrations of DNA were adjusted from 10 to 20 ng/mL.

**Table 18.1** Isolates

| IFM Number | Strain | Country (City) | Source (Remarks) | Phylogenetic species[a] | Accession no. (gp43) |
|---|---|---|---|---|---|
| *Paracoccidioides brasiliensis* | | | | | |
| IFM 41620 | Pb-9 | Brazil | Human patient | S1 | AB047690 |
| IFM 41621 | Pb-18 | Brazil | Human patient | S1 | AB047691 |
| IFM 41622 | Bt-2 | Brazil (Botucatu, São Paulo) | Human patient | S1 | AB304676 |
| IFM 41623 | Bt-3 | Brazil (Botucatu, São Paulo) | Human patient | S1 | AB304677 |
| IFM 41624 | Bt-4 | Brazil (Botucatu, São Paulo) | Human patient | S1 | AB047693 |
| IFM 41625 | Bt-7 | Brazil (Botucatu, São Paulo) | Human patient | S1 | AB304678 |
| IFM 41626 | Bt-9 | Brazil (Botucatu, São Paulo) | Human patient | S1 | AB047694 |
| IFM 41628 | B1183 | Brazil | Human patient | ND | ND |
| IFM 41629 | PbLev | Brazil | Human patient | S1 | AB304680 |
| IFM 41630 | B339 | Brazil | Human patient | S1 | AB304681 |
| (=CBS 372.73, =ATCC 32069) | | | | | |
| IFM 41631 | Recife | Brazil (Recife) | Human patient | S1 | AB304682 |
| IFM 41632 | Pb-HM-AOK | Japan (Tokyo)[b] | Human patient | S1 | AB047695 |
| IFM 41633 | Hachisuga | Japan (Fukuoka)[b] | Human patient | S1 | AB304682 |
| IFM 46215 | WAG | Japan (Osaka)[c] | Human patient | S1 | AB047696 |
| IFM 46240 | Tateishi | Japan (Ibaragi)[b] | Human patient | S1 | AB304684 |
| IFM 46464 | Bt-1 | Brazil (Botucatu, São Paulo) | Human patient | S1 | AB304685 |
| IFM 46465 | Pb-267 | Brazil | Mutant of Pb-9 | S1 | AB047692 |
| IFM 46466 | Pb-265 | Brazil | Mutant of Pb-9 | S1 | AB304686 |
| IFM 46467 | Recife-Pb-HC | Brazil (Recife) | Human patient | S1 | AB047699 |
| IFM 46468 | P-25 | Costa Rica (San Jose) | Human patient | ND | AB047698 |
| IFM 46470 | P-30 | Costa Rica (San Jose) | Human patient | ND | AB304688 |
| IFM 46930 | UMK | Japan (Chiba)[b] | Human patient | S1 | AB047697 |
| IFM 46463 | Tatu | Brazil (Botucatu, São Paulo) | Armadillo | S1 | AB047700 |
| IFM 47183 | PRT1 | Brazil (Botucatu, São Paulo) | Armadillo | S1 | AB047701 |
| IFM 47185 | PRT2 | Brazil (Botucatu, São Paulo) | Armadillo | S1 | AB047702 |
| IFM 47195 | D3LY1 | Brazil (Botucatu, São Paulo) | Armadillo | S1 | AB047813 |
| IFM 47217 | D4S1 | Brazil (Botucatu, São Paulo) | Armadillo | S1 | AB047704 |

(*continued*)

**Table 18.1** (continued)

| IFM Number | Strain | Country (City) | Source (Remarks) | Phylogenetic species[a] | Accession no. (gp43) |
|---|---|---|---|---|---|
| IFM 47228 | D4S9 | Brazil (Botucatu, São Paulo) | Armadillo | S1 | AB047703 |
| IFM 47247 | D4LIV1 | Brazil (Botucatu, São Paulo) | Armadillo | S1 | AB047705 |
| *Coccidioides immitis* sensu lato (*C. posadasii*) | | | | | |
| IFM 50993 | | USA | Human patient | – | – |
| *Histoplasma capsulatum* | | | | | |
| IFM 41329 | | USA | Human patient | – | – |
| *Blastomyces dermatitidis* | | | | | |
| IFM 41316 (=ATCC 26199) | | USA | Human patient | – | – |
| *Sporothrix schenkii* | | | | | |
| IFM 47068 | | Japan | Human patient | – | – |
| *Penicillium marneffei* | | | | | |
| IFM 41708 | | China | Bamboo rat | – | – |
| *Candida albicans* | | | | | |
| IFM 5740 | | Japan | Human patient | – | – |
| *Cryptococcus nenformans sensu lato* | | | | | |
| IFM 5830 | | Japan | Human patient | – | – |

*IFM* Institute of Food Microbiology, Chiba University, the former name of the Medical Mycology Researc Center, and deposited as the official abbreviation of the world culture collection of pathogenic fungi and actinomycetes

[a]Phylogenetic species was estimated from *gp43* sequence

[b]The patient was infected in Brazil

[c]The patient was infected in Paraguay

*ND* Not determined

DNA extracted from a paraffin-embedded tissue sample of PCM and an ethanol-fixed sputum sample was extracted with a DEXPAT kit (TAKARA BIO INC.) and was also used in the LAMP assay.

#### 18.3.2.3 Detection of *gp43* by PCR

A total volume of 25 μL was used for all PCR reactions. Fifty nanograms per milliliter of DNA extracts were added to 2.5 μL of Ex Taq™ buffer in the kit (Ex Taq™, TAKARA BIO INC.) containing 4.5 mM MgSO4, 2 μL (2.5 mM each) dNTP mixture in the kit (ExTaq™, TAKARA BIO INC.), 2 μL each 10 pM primer set of F3 5′-TCA CGT CGC ATC TCA CAT TG-3′ encoding from 391st to 410th and B3 5′-AAG CGC CTT GTC CAA ATA GTC GA-3′ designed from the complementary sequence from 718th to 740th correspondent to gp43 sequence at GenBank U26160 and 0.0625 μL (5 units/μL) TaKaRa Ex Taq™ polymerase in the kit (Ex Taq™, TAKARA BIO INC.). Reaction mixtures were subjected to denaturation at 94°C for 1 min, followed by 30 cycles of amplification at 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min and a final extension at 72°C for 10 min, in a PCR Thermal Cycler MP (TAKARA BIO INC.). PCR products were separated by electrophoresis on 1.0% agarose gels in TAE buffer (40 mM Tris-base, 20 mM acetic acid, 1 mM EDTA), stained with ethidium bromide, and visualized by UV transillumination. DNA strands obtained from the PCR were processed for direct sequencing with ABI Prism 3,100 (Applied Biosystems, Foster City, CA., USA) to confirm the sequence of *gp43* (Sano et al. 1998–1999).

#### 18.3.2.4 LAMP Method for *gp43*

Briefly, the LAMP method used in the present study detects the *gp43* gene with a combination of F3, B3, FIP, and BIP primers designed from the partial sequence of *gp43* (GenBank accession number U26160) by a registration system primer designing website (FUJITSU Ltd., Tokyo, Japan: "LAMP PIMER EXPLORER" website in "Netlaboratory" homepage http://venus.netlaboratory.com/partner/lamp/index.html). These primers recognize a partial sequence of *gp43*.

The primer sequences were as follows: F3, used in the species specific forward primer; B3, used in the species-specific reverse one; FIP, 5′-TGG CTC CAG CAA TAG CCA CCC GTC AAG CAG GAT CAG CAA T-3′ designed from the forward sequence of 425th to 445th and the complementary sequence of 464th to 485th; and BIP: 5′-CAT GTC AGG ATC CCG ATC GGG CCT TGT ACA TAT GGC TCT CCC T-3′ designed by the forward sequence from 648th to 668th and the complementary sequence from 691st to 712th. The annealing sites of the primers are shown in Fig. 18.3.

One micro liter of 10 ng/mL DNA template and 40 pmol each of the FIP and BIP primers and 5 pmol each of the F3 and B3 primers were mixed with 12.5 μL of 2 reaction mix in the kit (Loop AMP, Eiken Chemical Co., Ltd., Tokyo, Japan) in a

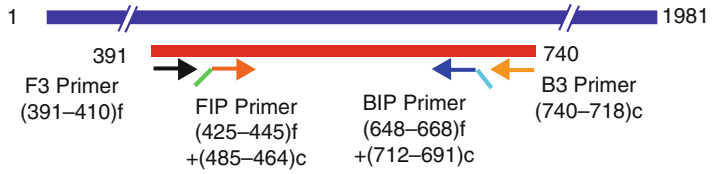


**Fig. 18.3** Primer map for the LAMP method of detecting *gp43* from *P. brasiliensis*

final volume of 23.0 μL. DNA mixtures were incubated at 63°C for 60 min. The reaction was stopped by heating the mixture at 80°C for 2 min to inactivate the enzyme of LAMP amplification. Detection limits of the LAMP method were evaluated with serial dilutions of DNA from isolate IFM 46930.

As the positive control attached with the kit and a negative control consisted of DW and other fungal DNAs, *C. immitis*, *H. capsulatum*, *B. dermatitidis, S. schenckii*, *P. marneffei*, *C. albicans*, and *Cr. neoformans* were used. In addition, DNAs extracted from a paraffin-embedded tissue sample, and an ethanol-fixed sputum were reacted at 63°C for 60 and 120 min.

In addition, time-dependent increases in levels of DNA products by LAMP were monitored by real-time-PCR (Rotor-Gene, RG2000, NIPPN/Techno Cluster, Inc., Tokyo, Japan) for as long as 70 min at 63°C with *P. brasiliensis* isolates IFM 41630 and IFM 46215.

## 18.4 Results

The PCR products amplified with the primer set; F3 and B3 showed species specificity for *P. brasiliensis*. The detection limit of the PCR was 100 fg of fungal genomic DNA (data not shown). Other related species, such as *C. posadasiis* (not shown), *H. capsulatum*, and *B. dermatitidis* or important pathogenic fungi; *S. schenckii*, *P. marneffei*, *Ca. albicans*, and *Cr. neoformans* were negative (Fig. 18.4).

All partial sequences of *gp43* consisted of 339 bps and were correspondent to their accession numbers, except for isolate IFM 41628 (not done).

The specific DNA banding pattern of *P. brasiliensis* was detected in the clinical and nine-banded armadillo derived isolates by LAMP. No DNA band was observed in negative control isolates of *C. posadasii*, *H. capsulatum*, *B. dermatitidis*, *S. schenckii*, *P. marneffei*, *C. albicans*, and *Cr. neoformans* (Fig. 18.5). The detection limit of LAMP for *gp43* was also 100 fg of fungal genomic DNA.

The incubation procedure at 63°C for 60 min was not sufficient for detection of *gp43* from DNA extracted from paraffin-embedded tissue sample or sputum infected with PCM (data not shown). The DNA from a paraffin-embedded tissue and sputum from different patients yielded the same ladder band yielded by fungal DNAs *via* LAMP at 63°C for 120 min (Fig. 18.6a, b).

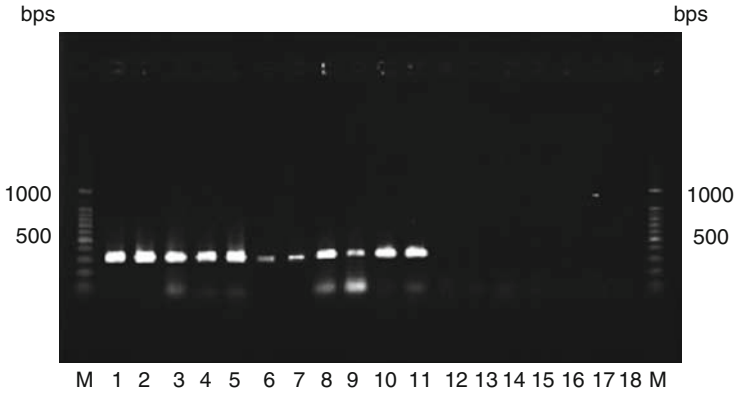


**Fig. 18.4** Amplification of the *gp43* gene by PCR with primers F3 and B3. All DNA derived from *P. brasiliensis* isolates (line 1–11) were uniformly positive. 12: *Ca. albicans,* 13: *H. capsulatum*, 14: *B. dermatitidis*, 15: *P. marneffei*, 16: *S. schenckii*, 17: *Cr. neoformans*, and 18: *C. immitis* (*C. posadasii*) were negative

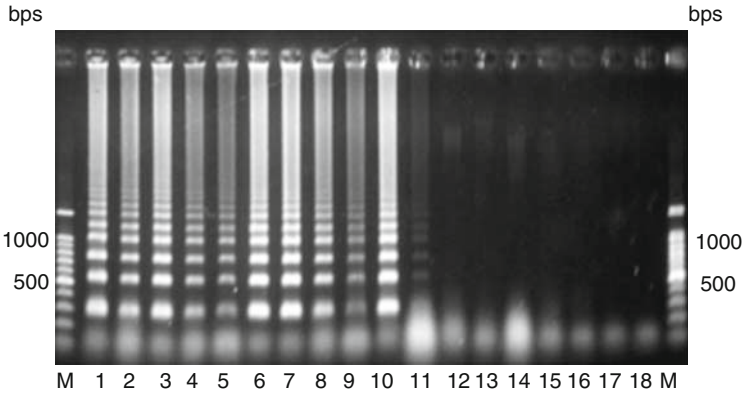


**Fig. 18.5** Amplification of the *gp43* gene by the LAMP methods. All DNA derived from *P. brasiliensis* isolates (line 1–11) were uniformly positive. 12: *Ca. albicans,* 13: *H. capsulatum*, 14: *B. dermatitidis*, 15: *P. marneffei*, 16: *S. schenckii*, 17: *Cr. neoformans*, and 18: *C. immitis* 18: *C. immitis* (*C. posadasii*) were negative

The LAMP reaction reached a plateau after incubation at 63°C for 45 min, so far, as monitored by real time- PCR (Fig. 18.7). The positive control provided with the kit reached a plateau at 15 min, and the negative one did not show increase of fluorescence level. DNAs from other fungal species did not increase the fluorescence level (data not shown). The LAMP reaction of DNA from isolate IFM 46215 reached a plateau at 63°C for 45 min and those of IFM 41622 was 50 min.

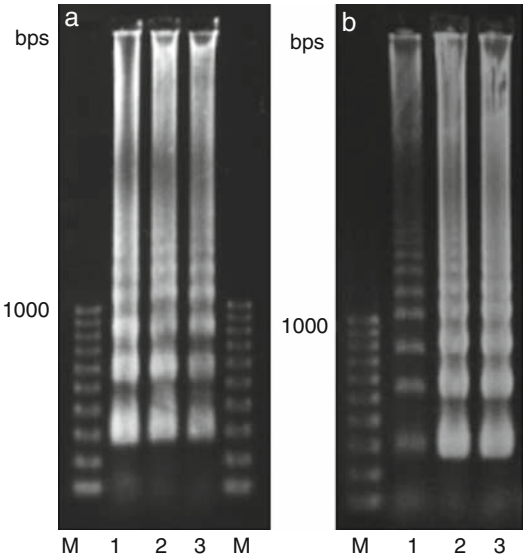


**Fig. 18.6** (**a**) Amplification of the *gp43* from paraffin embedded tissue sample by the LAMP methods. M: Marker, 2: DNA from the paraffin embedded tissue sample. 3 and 4: Fungal DNA of *P. brasiliensis*. (**b**) Those from sputa. M: marker, 2: DNA from the sputum, 3 and 4: Fungal DNA of *P. brasiliensis*

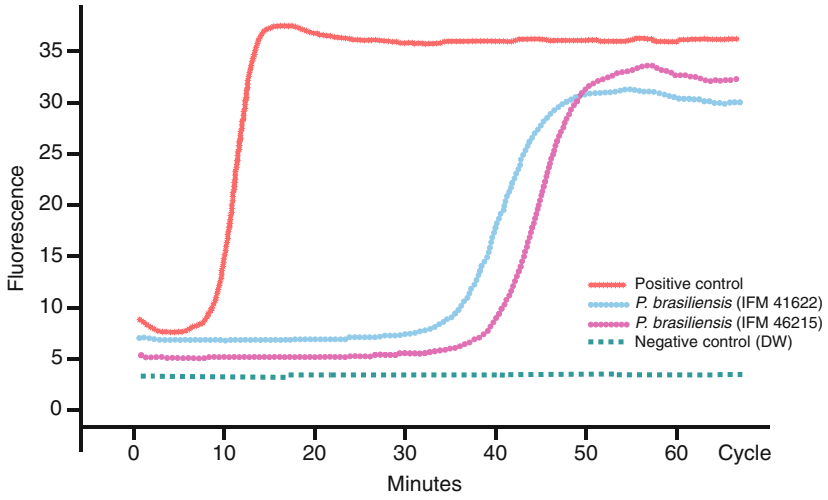


**Fig. 18.7** LAMP reaction monitored by real-time-PCR. The negative control with other fungal DNAs, *C. immitis* (*C. posadasii*), *H. capsulatum*, *B. dermatitidis*, *S. schenckii*, *P. marneffei*, *Ca. albicans*, and *Cr. neoformans* were as the same as DW

## 18.5 Comments and Opinions

The LAMP method provides for more rapid detection of *gp43* than nested PCR. LAMP required only 3 h from DNA extraction to identification, whereas nested PCR required 12 h when we tested.

LAMP methods are also advantageous because it can be applied to clinical material, such as paraffin-embedded tissue and sputum samples for retrospective study (Endo et al. 2004; Tatibana et al. 2009). Even in clinical samples, the time required for diagnosis was less than 4 h.

The LAMP method is not only convenient for identification of *P. brasiliensis*, but also for diagnosis of PCM, especially for identification of *P. brasiliensis* and diagnosis of PCM outside of the endemic areas, such as European countries and Japan. Patients in endemic areas are sometimes misdiagnosed as having a malignant tumor because of a shadow on the chest X-ray and granulomatous inflammation of infected tissue. Therefore, most PCM of patients in Japan are being diagnosed on the basis of histopathological findings (Endo et al. 2004). The LAMP method could be applied for PCM diagnosis in such cases without isolation of the fungus.

Application of real-time-PCR to the LAMP method should shorten the time for obtaining the results within a couple of hours, because electrophoresis is not required. While analysis of LAMP amplification products by agarose gel electrophoresis takes approximately 3 h, LAMP in connection with real-time-PCR takes only 2 h.

According to the manufacturer's protocol, LAMP products can be detected by optical density under UV light. However, we do not recommend this method. Some of pseudo reactions showing smear-like amplification products also became positive. Furthermore, we do not have any experience to react as a smear-like amplification in the real-time PCR method. Uncertainty of the reaction also could not be removed. Therefore, LAMP products should be visualized by agarose gel electrophoresis.

In addition, the reaction does not require a special thermo cycler system. A styrofoam box with warm water like that of a hot coffee temperature is one sign of a good apparatus. It suggested that the method is useful in field hospitals.

This method will be important for detecting specific genes in highly pathogenic or rare emerging fungal infections which require care and time-consuming culturing procedures.

However, because of extremely higher sensitivities to detecting genes by the LAMP methods, it should be meaningless to apply the LAMP methods to *Candida* species that exist as common fungal flora in oral or body surface, to *Aspergillus* species and/or to other causative agents for the emerging fungal infections habitat popular in soil or environments. It should be impossible to judge the results whether it is environmental contaminations or real infectious propague. In addition, we would like to avoid to give a comment on the report by Inácio et al (2008).

## 18.6 LAMP Method for Identifications of *O. gallopava*

We also applied the LAMP method to detection of the species-specific gene of *O. gallopava*; a species of dematiaceous fungi recognized as a causative agent of zoonotic and emerging fungal infections. The fungal specie shows excellent growth at 42°C (Fig. 18.8a), and is able to grow up to 45°C or more.

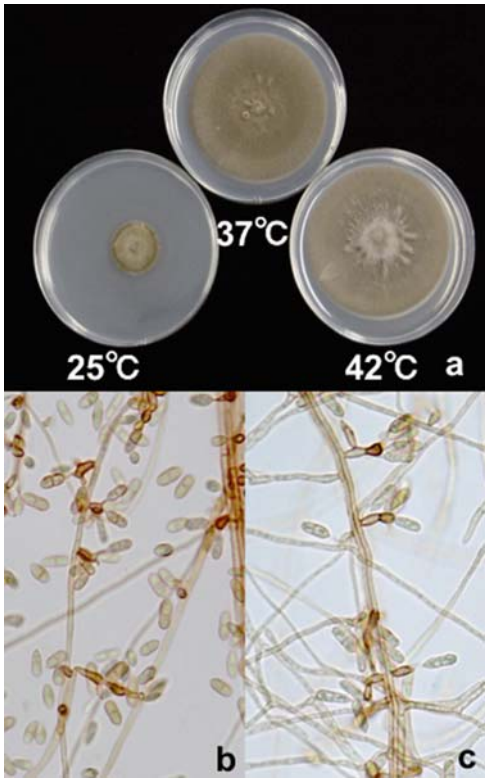


**Fig. 18.8** (**a**) Colonies of *O. gallopava* cultured on potato dextrose agar plate at 25, 37 and 42°C for 8 days, (**b** and **c**) clavate conidia under microscopy, x400

It affects the central nervous system and respiratory tracts of humans, birds and cats and is required to be differentiated from SARS and highly pathogenic bird flu. Clavate conidia (Fig. 18.8b and c) are virulent to experimentally infected mice (Ohori et al. 2006; Yarita et al. 2007).

We designed *O. gallopava* species-specific primer sets to aid in its identification by the LAMP method based on the D1/D2 domain of the LSU rDNA sequence.

The primer set for *O. gallopava* was designed based on the sequence of D1/D2 LSU rDNA of *O. gallopava* (accession number AB125281 in GenBank) with a comparison of 21 species of dematiaceous fungi obtained from the present study and from 108 sequences in GenBank database. The primer sequences were as follows: OgF3: 5′-AGG GAG TCT CGG GTT AAG GG-3′ encoding from the 391st to the 410th, and OgB3: 5′-CAT TCC CTT CGT CTT TGT CC-3′ corresponding to the complementary sequence from the 718th to the 740th of AB125281 and were species-specific for *O. gallopava* (Fig. 18.9). FIP; 5′-ACT CGA CTC GTC GAA GGG GCA GAG GGT GAG AGT CCC GT-3′ designed by the forward sequence of 425th to 445th and the complementary sequence of 464th to 485th, and BIP; 5′-ACT GGC CAG AGA CCG ATA GCG TGA CTC TCT TTT

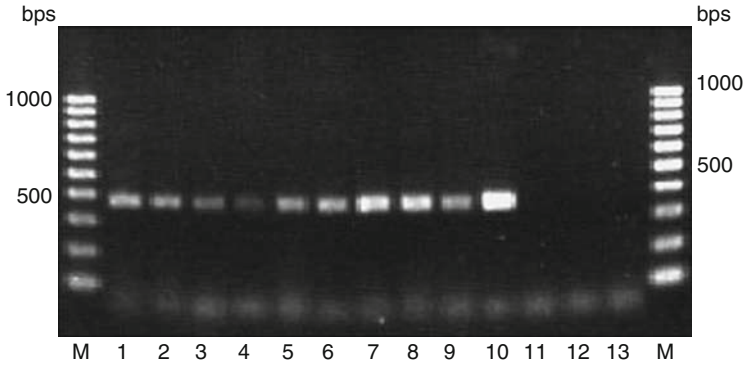


**Fig. 18.9** Species specific PCR for *O. gallopava*. M: Marker, 1–10: *O. gallopava*, 11: *O. gamsii*, 12: and 13: *O. tsawytschae*. The related species such as *O. constricta, O. humicola, Alternaria alternata, Arthrobotrys javanica, Bipolaris* sp., *Bipolaris specifera, Cladophialophora bantiana, C. carrionii, Curvularia geniculata, Cu. lunata var. lunata, Cu. senegalensis, Exophiala alcalophiala, E. dermatitidis, E. jeanselmei, E. moniliae, E. spinifera, Fonsecaea pedrosoi, Phialophora verrucosa, Rhinocladiella atrovirens, Scolecobasidium terreum* were negative

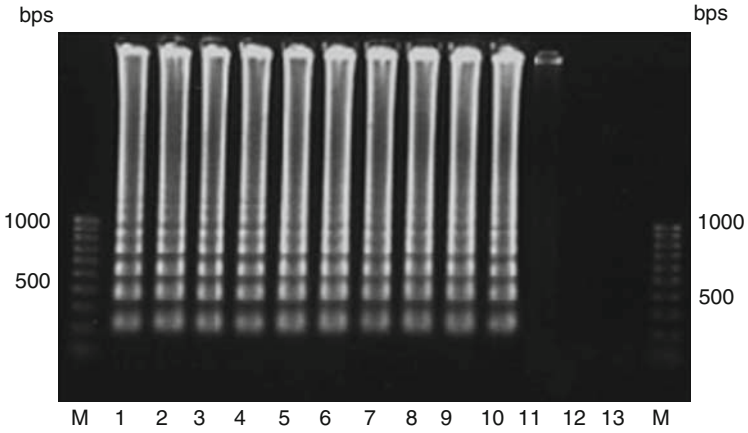


**Fig. 18.10** Species specific loop mediated isothermal amplification method (LAMP) for *O. gallopava*. M: Marker, 1–10: *O. gallopava*, 11: *O. gamsii*, 12: and 13: *O. tsawytschae*. The related species such as *O. constricta, O. humicola, Alternaria alternata, Arthrobotrys javanica, Bipolaris sp., Bipolaris specifera, Cladophialophora bantiana, C. carrionii, Curvularia geniculata, Cu. lunata var. lunata, Cu. senegalensis, Exophiala alcalophiala, E. dermatitidis, E. jeanselmei, E. moniliae, E. spinifera, Fonsecaea pedrosoi, Phialophora verrucosa, Rhinocladiella atrovirens, Scolecobasidium terreum* were negative

CAA AGT GC-3′ designed by the forward sequence from 648th to 668th and the complementary sequence from 691 st to 712 nd of AB125281.

The LAMP method successfully detected the gene from both the fungal DNA derived from experimentally infected brains and spleens of mice and environmental

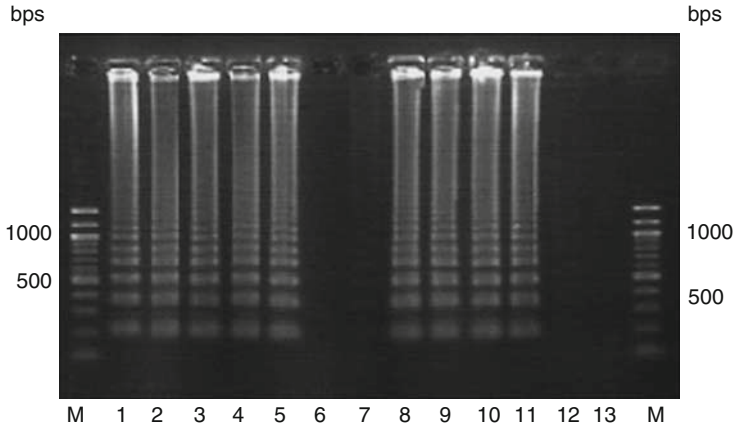


**Fig. 18.11** Detection of *O. gallopava* gene from the experimentally infected brains and spleens of mice by LAMP method. M: Marker, 1–5: brain tissue of mice infected with *O. gallopava*. 6: blank, 7–11: spleen tissue of mice infected with *O. gallopava*. 12 and 13: negative control DNA from demateaceous fungi

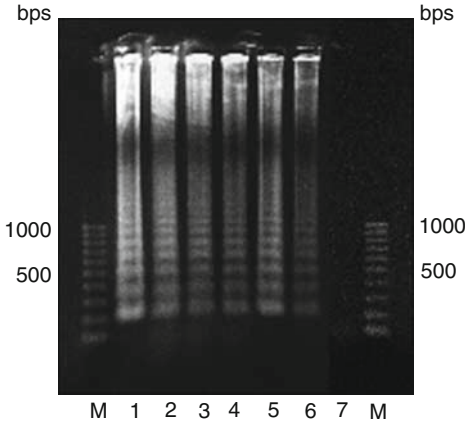


**Fig. 18.12** DNA pattern by loop madiated isothermal amplification method (LAMP) specific for *O. gallopava* using 20 pg of fungal DNA. M: Marker, 1 and 6: a clinical isolate, 2–5: hot spring isolates, 7: a negative control using distilled water for a template

isolates (Fig. 18.10–18.12),which will help to differentiate *O. gallopava* infection from other important avian zoonoses (Ohori et al. 2006; Yarita et al. 2007).

## 18.7 Conclusion and Future Line of Research

In conclusion, LAMP method for PCM and *O. gallopava* seemed to be useful for fungal identification, diagnosis or retrospective study with advantage in the quickness and simplicity procedure, but require strictly controlled environments. It could be applicable for clinical identification of fungi and diagnosis of fungal

diseases caused by level 3 biohazards, such as coccidioidomycosis, histoplasmosis, blastomycosis, and infection of *Penicillium marneffei*, which generally require care and time consuming culturing procedures, and causative agent for emerging fungal infections.

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