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Full Length Research Paper

Development and evaluation of loop-mediated isothermal amplification (LAMP) for the rapid diagnosis of *Candida parapsilosis*

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The *Candida parapsilosis* family has emerged as a major opportunistic and nosocomial pathogen. It causes multifaceted pathology in immuno-compromised and normal hosts, notably low birth weight neonates. In the present study, a novel method, known as loop-mediated isothermal amplification (LAMP), was described for the rapid and specific detection of the species, using primer sets derived from the 5.8 S ribosomal RNA gene of *C. parapsilosis* (internal transcribed spacer 2, ITS2). Amplification products can be detected macroscopically by visual inspection in vials using SYBRGreen I as well as by electrophoresis on agarose gel. The LAMP assay resulted in specific amplification of the ITS2 of *C. parapsilosis* using pure cultures after a 45-min reaction at 65°C; no cross-reactivity with other fungi including other *Candida* species was observed. The detectable DNA limit was 0.01 pg fungal DNA per reaction, equivalent to 3.74 × 10^{-3}cfu/ml. In addition, specific amplification was achieved using 30 proven *C. parapsilosis* strains from patients samples. The method provides a powerful tool for rapid diagnostics in the clinical laboratory, and has potential for use in ecological studies.

Key words: Loop-mediated isothermal amplification, diagnosis, *Candida parapsilosis.*

INTRODUCTION

Over the past decade, the incidence of *Candida parapsilosis* has dramatically increased. In fact, reports indicate that *C. parapsilosis* is often the second most commonly isolated *Candida* species from blood cultures (Almirante et al., 2006; Brito et al., 2006), and *C. parapsilosis* even outranks *Candida albicans* in some European (Nakamura and Takahashi, 2006), Asian (Nakamura and Takahashi, 2006; Ng et al., 2001) and South American (Medrano et al., 2006) hospitals. This species has emerged as an important nosocomial pathogen, with clinical manifestations including fungemia, endocarditis, endophthalmitis, septic arthritis and peritonitis, all of which usually occur in association with invasive procedures or prosthetic devices. Outbreaks of *C. parapsilosis* infections have been caused by contamination of hyperalimentation solutions, intravascular pressure monitoring devices, and ophthalmic irrigating solution. Experimental studies have generally shown that *C. parapsilosis* is less virulent than

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C. albicans or *Candida tropicalis*. However, characteristics of *C. parapsilosis* that may relate to its increasing occurrence in nosocomial settings include frequent colonization of the skin (Bonassoli et al., 2005), particularly the subungual space, and an ability to proliferate in glucose-containing solutions, with a resultant increase in adherence to synthetic materials (Alonso-Valle et al., 2003).

Traditionally, *C. parapsilosis* strains have been identified based on morphological, physiological and biochemical characteristics (Van Asbeck et al., 2009). These methods are laborious and time consuming. Currently, matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) MS is reported as a reliable, rapid and simple technique for the identification of the *C. parapsilosis* group (Quiles-Melero et al., 2012). However, MALDI-TOF MS requires expensive equipment, which impedes it as an attractive tool for the routine of a clinical microbiology laboratory. Molecular methods based on the analysis of polymorphism in the DNA region that encodes the ribosomal RNA genes (5S, 5.8S, 18S and 26S) (Kurtzman and Robnett, 1998; Nosek et al., 2002; Sofair et al., 2006) and the non-coding internal transcribed spacers (ITS)(Cadez et al., 2002; Sabate et al., 2002) and IGS (Intergenic Spacer) regions (Diaz et al., 2000; Naumov et al., 2003) are being successfully used for the identification of many yeast species. Recently, developed molecular techniques may facilitate the continued exploration of the epidemiology and pathogenesis of *C. parapsilosis* infections. However, all have been developed based on cultured material, and require a fully equipped molecular laboratory. Thus, there is still a need for a rapid and simple technique that is able to deliver an unambiguous identification within a single day.

Loop-mediated isothermal amplification (LAMP) is a novel nucleic acid amplification method, which relies on autocycling strand displacement DNA synthesis performed by the *Bst* DNA polymerase large fragment (Mori et al., 2001; Nagamine et al., 2002; Notomi et al., 2000). The amplification products are stem-loop DNA structures with several inverted repeats of the target and cauliflower-like structures with multiple loops. LAMP has the following characteristics: (i) all reactions can be conducted under isothermal conditions ranging from 60 to 65°C by using only one type of enzyme; (ii) the specificity of the reaction is extremely high because it uses four primers recognizing six distinct regions on the target DNA; (iii) amplification can be performed in a shorter time than amplification by PCR because there is no time loss due to thermal cycling; and (iv) it produces extremely large amounts of amplified products and enables simple detection methods such as visual judgment by the turbidity or fluorescence of the reaction mixture, which is kept in the reaction tube (Mori and Notomi, 2009). With all these characteristics, LAMP of target DNA has emerged as a powerful tool to facilitate point-of-care genetic testing at the bedside. Recently, Nagamine et al. (2002) reported that when two more primers, termed loop primers, were added, the LAMP reaction time could be even less than half of that for the original LAMP method. In their procedure, six primers recognized eight distinct regions on the targeted DNA. In the present study, we introduced LAMP diagnostics for *C. parapsilosis*. The sensitivity, specificity and applicability of this method for *C. parapsilosis* from patient samples were evaluated. It is believed that the rapid detection and confirmation of *C. parapsilosis* in clinical specimens is essential for efficient management.

MATERIALS AND METHODS

Strains

Thirty proven strains of *C. parapsilosis* isolated from patients, 5 isolates of other reference strains including *C. albicans*, *C. tropicals*, *Candida glabrata*, *Candida krusei* and *Cryptococcus neoformans* and one *C. parapsilosis* type strain ATCC 22019 were used in this study. The 30 strains of *C. parapsilosis* and the 5 other reference strains were all collected in Department of Laboratory Medicine, The First Affiliated Hospital of Sun Yat-sen University during the period of January 2010 to December 2012. Cases from patients were confirmed by routine and molecular identification methods. All the isolates were cultured on Sabourand dextrose agar (SDA) at 37°C. Inoculated plates were examined after 48 h of incubation. Identification of *Candida* species were based on VITEK 2 system (bioMérieux, Marcy l'Etoile, France) and further identified by 18S rRNA gene sequencing as described by Zheng et al. (2013).

DNA extraction

Candida species were grown on SDA plates for 24 to 48 h at 30°C. Single colonies were inoculated into 200 ml of YPD broth (1% yeast extract, 2% peptone, 2% glucose) and incubated in a shaking water bath at 200 rpm and 30°C for 36 h. DNA was extracted from this culture by adaptation of the Lyticase-based method (10KU, Sigama, USA). DNA concentrations and A260/A280 ratios were determined using a spectrophotometer Lambda 1A (Perkin-Elmer, USA). An A260/A280 ratio of 1.8-2.1 was considered acceptable.

Design of LAMP primers

The target gene of the LAMP was the 5.8 S ribosomal RNA gene of *C. parapsilosis* (internal transcribed spacer 2, ITS2). The binding sites of all primer sets are located within the target gene and were designed by using PrimerExplorer software V4 (Eiken Chemical Co. Ltd.) in the database under the Accession No. KF313207. A set of sixLAMP primers was selected as follows: outer primers (F3 and B3), a forward inner primer (FIP), a backward inner primer (BIP) and loop primers (loop F and loop B) (Table 1).

LAMP reaction

The LAMP reaction was performed with a Loop amp DNA amplification kit (Eiken Chemical Co., Ltd., Tochigi, Japan). A reaction mixture (25 µl) containing 1.2 µM each inner primer (FIP and BIP), 0.2 µM each outer primer (F3 and B3), 0.8 µM each loop primer (F and B), 0.8 mM dNTPs, 1M betaine (Sigma), 1×ThermoPol Buffer, 4 mM $MgSO_4$, 8 U of Bst DNA large fragment polymerase (New England Biolabs), with 2 µl of crude DNA extract

Table 1. Sequences of primers used in the LAMP assay.

Primer name	Sequence(5'→ 3')
Forward outer (F3)	AACGAGAGTATCACTCACTAC
Backward outer (B3)	TCAACAATGGATCTCTTGGT
Forward inner primer (FIP)	ATTGCGCCCTCTGGTATTCCCAAACACAACGTGTTTGAGA
Backward inner primer (BIP)	GTGCGTTCAAAGATTCGATGATTCTCTCGCATCGATGAAGAAC
LF	CCTGTTTGAGCGTCATTTCCT
LB	ACGGAATTCTGCAATTCACATTACG

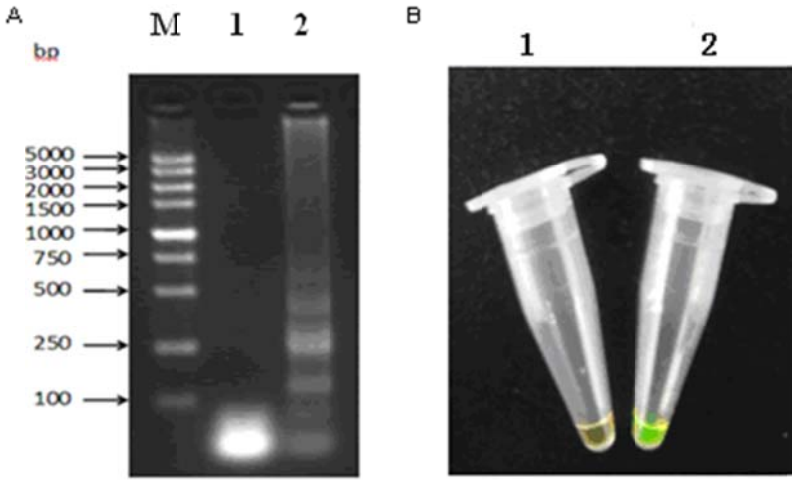

Figure 1. Identification specificities of the LAMP assay for *C. parapsilosis*. (A) Electrophoretic analysis of LAMP amplified products. Lane M, 100-bp ladder used as a size marker; Lane 1, negative control; Lane 2, *C. parapsilosis* ATCC22019. (B) Visual inspection of LAMP amplified products. Tube 1, negative control; Tube 2, *C. parapsilosis* ATCC22019.

as the template and the specified amounts of DNA lysates was incubated at 65°C for 45 min and was heated at more than 80°C for 2 min to terminate the reaction. Positive and negative controls were included in each run, and all precautions to prevent cross contamination were observed.

PCR reaction

To compare the detection sensitivities of LAMP and PCR, PCR using F3 and B3 primers which amplify a 446-bp product was carried out in a total reaction volume of 25 µl containing 1 µl of the fungal DNA, 2 µl of a pair of appropriate primers (0.1 mM), 2 µl dNTPs mixture (0.8 mM), 2.5 U ExTaq™ DNA polymerase ((TaKaRa, Shiga, Japan) with the corresponding polymerase buffer were mixed. PCR conditions consisted of an initial denaturation of 94°C for 4 min and 30 cycles of 94°C for 60 s, 58°C for 60 s, 72°C for 90 s and a final extension of 72°Cfor 4 min in a DNA thermal cycler 9700 (Applied Biosystems, Foster City, CA). The amplified products (4µl) were then analyzed by 1% agarose gel.

Analysis of LAMP products

Amplified products were analyzed by electrophoresis on 1% agarose gels, stained with ethidium bromide and photographed. A 100-bp DNA ladder was used as the molecular weight standard. LAMP amplicons in the reaction tube were directly detected with the naked eye by adding 1.0 µl of 1/10-diluted original SYBR Green I (Molecular Probes Inc.) to the tube and observing the color of the solution. The solution turned green in the presence of a LAMP amplicon, while it remained orange with no amplification. The sensitivities of electrophoresis and SYBR Green I inspection with the naked eye were compared by using seriallydiluted LAMP products.

RESULTS

Specificity of LAMP assay

The specificity of LAMP was tested using fungal DNA extracted from *C. parapsilosis* ATCC22019, 5 proven isolates of *C. parapsilosis* and 5 isolates of non-*C. parapsilosis*, including *C. albicans*, *C. tropicals*, *C. glabrata*, *C. krusei* and *Cryptococcus neoformans*. After incubation at 65°C for 45 min, all the *C. parapsilosis* isolates were positively detected, whereas no cross-reactivity with other fungi including other *Candida* species such as *C. albicans* was observed (Figure 1). The products

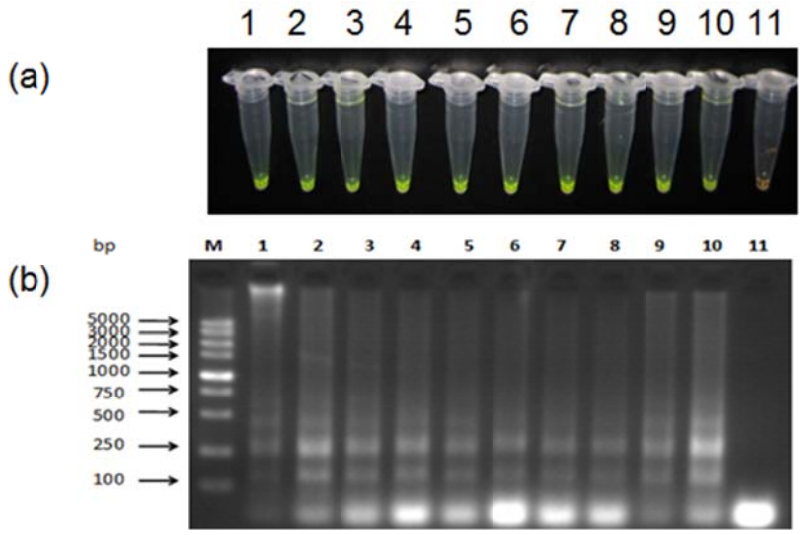

Figure 2. Sensitivities of visual inspection (a) and electrophoretic analysis (b) of LAMP amplified products. The number above each tube or lanes 1-11 represents the dilution of the LAMP product: 1, no dilution; 2-11: 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10} dilutions of DNA templates from *C. parapsilosis*.

of the LAMP reaction could be detected by electrophoresis on 1% agarose gels and showed ladder-like patterns (1). The products could also be made visible to the naked eye directly in Eppendorf vials or under UV transillumination after adding SYBR Green I dye. Positive reactions showed bright green fluorescence, whereas negative reactions remained light orange. These results indicate that the LAMP method is highly specific for *C. parapsilosis* in the study.

Sensitivity of the LAMP assay

To assess the detection sensitivity of the LAMP assay for the detection of *C. parapsilosis*, the reaction was tested using 1-µl tenfold serial dilutions of fungal DNA (1 µg/ml) and compared with the PCR assay. The LAMP reaction was able to detect *C. parapsilosis* up to 0.01 pg fungal DNA per reaction, equivalent to 3.74×10^{-3} cfu/ml. However, PCR could only detect *C. parapsilosis* up to 0.1 pg fungal DNA per reaction. LAMP amplification products were analyzed visually by addition 1 µl SYBR Green I and by 2% agarose gel electrophoresis (Figure 2). The results indicate a tenfold higher sensitivity of LAMP than the standard PCR method.

Identification of *Candida* strains isolated from clinical samples

Clinical samples were first discriminated by VITEK 2 system and further identified by 18SrRNA gene sequencing, and then assessed by LAMP established in this study. The results showed that all the 30 proven *C. parapsilosis* strains were detected, suggesting that the established LAMP assay for *C. parapsilosis* represented a great consistency with conventional PCR and VITEK 2 system (Figure 3).

DISCUSSION

LAMP is a powerful innovative gene amplification technique providing a simple and rapid tool for early detection and identification of microbial diseases. In the present study, we developed and evaluated the LAMP assay, exemplified by the detection and identification of *C. parapsilosis* in DNA from pure cultures. The LAMP assay is a simple detection tool in which the reaction is performed in a single tube by mixing the thermopol buffer, primers, and *Bst* DNA polymerase, and incubation of the mixture at 65°C for 45 min. The LAMP reaction is done under isothermal conditions and it does not require expensive equipment. The only equipment needed for the LAMP reaction is a regular laboratory water bath or a heating block that can provide a constant temperature of65°C. Moreover, the amplification efficiency is extremely high because there is no time loss because of thermal cycling and inhibition reactions at later stages are less likely to occur unlike in standard PCR. In addition, LAMP amplifies DNA to higher concentrations than PCR making it convenient for visualizing the products after addition of SYBR Green I without gel electrophoresis. Hence, the LAMP assay could be developed into a field

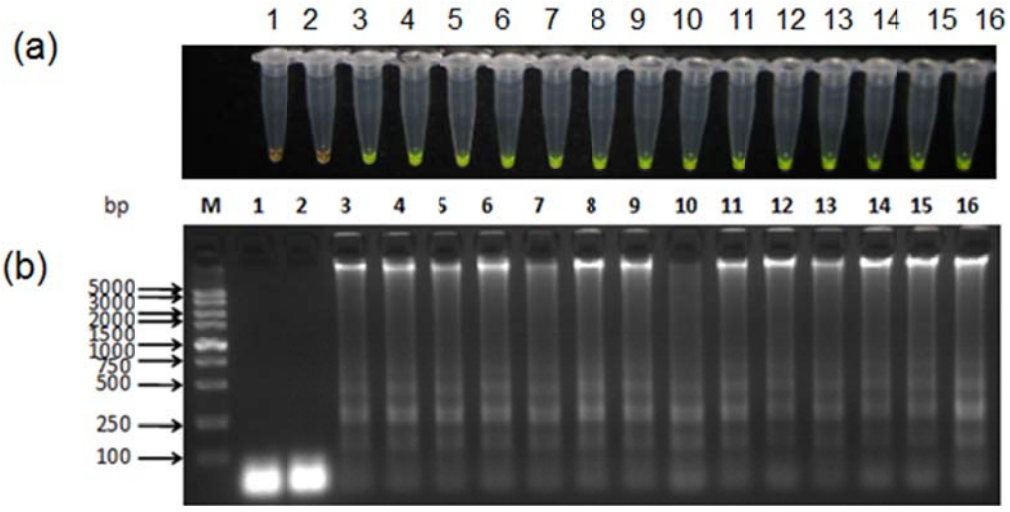

Figure 3. Identification of *Candida* strains isolated from clinical samples. (a) Electrophoretic analysis of LAMP amplified products. (b) Visual inspection of LAMP amplified products. The number above each Lane 1 represents *Candida albicans* strain; Lane 2 represents *Candida tropical* strain; Tube or Lane 3-16 represent DNA templates from proven *C. parapsilosis* strains.

test and made available to empower active efforts to identify *C. parapsilosis*.

During the past decade, various nucleic acid amplification based methods have been developed to address the need for rapid and sensitive diagnosis of *C. parapsilosis* (Burton et al., 2011). These methods require either precision instruments for the amplification or elaborate methods for detection of the amplified products, which are the major obstacles to wide use of these methods in relatively small scale clinical laboratories (Carolis et al., 2014; Del et al., 2011; Hays et al., 2011). In this regard, the LAMP-based assay developed in this study has the advantages of rapid reaction, simple operation and easy detection.

In this study, the LAMP method detecting *C. parapsilosis* was found to be highly sensitive, as it could detect *C. parapsilosis* up to 0.01 pg fungal DNA per reaction, equivalent to 3.74×10^{-3} cfu/ml, whereas by PCR, the detection of *C. parapsilosis* was possible up to 0.1 pg fungal DNA per reaction. This indicates that the sensitivity of LAMP is ten times more than that of the standard PCR. This increased sensitivity makes LAMP a better choice than PCR for the detection of *C. parapsilosis* in cases where lower fungal concentrations are expected.

Identification of the species of *C. parapsilosis* isolates is another critical requirement for clinical laboratories. In the present study, the results showed that all the 30 proven *C. parapsilosis* isolates from clinical samples were detected by the LAMP assay, suggesting that the established LAMP assay for *C. parapsilosis* represented a great consistency with conventional PCR and VITEK 2 system. The conventional biochemical tests for identification of *C. parapsilosis* are relatively time-consuming. The LAMP-based assay can identify *C. parapsilosis* in 80 min: 30 min for DNA extraction, 45 min for the LAMP reaction and 1 min for detection.

In conclusion, the LAMP method described in this study represents a new sensitive, specific and rapid protocol for the detection of *C. parapsilosis*. Due to its easy operation without sophisticated equipment, it will be simple enough to use in small-scale hospitals, primary care facilities and clinical laboratories in developing countries if the remaining issues such as nucleic acid extraction and cross-contamination controls are addressed. Our next direction in developing this promising method for wider clinical use would be to detect *C. parapsilosis* in clinical specimens such as blood, urine and sputum.

Conflict of Interest

The author(s) have not declared any conflict of interests.

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