IJBPAS, January, 2016, 5(1), Special Issue: 572-581
ISSN: 2277–4998

International Journal of Biology, Pharmacy and Allied Sciences (IJBPAS)
'A Bridge Between Laboratory and Reader'
www.ijbpas.com

LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (LAMP) FOR RAPID DETECTION OF *CANDIDA ALBICANS IN SAMPLE OF PETS*

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ABSTRACT

Candidiasis is the most common opportunistic fungal disease in animals and humans. The timely diagnosis candidiasis is one of the problems has been detected. The purpose of this study correct and rapid diagnosis molecular *Candida albicans* in the samples of peds that can help measure suitable treatment. : LAMP test optimized on the basis of alfa INT1 gene and then limited of detection (LOD) and specificity evaluated . In the specificity test with selected primers no product was not observed with different DNA samples, that indicating the high specificity of the primers and the LOD of LAMP tests was found to be approximately 100 CFU. However, LAMP test is a rapid, sensitive and specific method and also low cost for diagnosis of systemic candidiasis in samples such as pets in laboratory centers.

Keywords: LAMP, molecular diagnosis, *Candida albicans*

INTRODUCTION

Candidiasis is the most common opportunistic fungal disease in animals and humans[1] . candidiasis is caused by Candida yeasts, . *Candida albicans* is the most virulent and prevalent species, followed by the non-albicans *species C. tropicalis, C. glabrata, C. parapsilosis, C. krusei, C. dubliniensis*, and others [2,3] . *Candida albicans* is, a dimorphic fungus, the most common pathogen of humans and animal [4] . This disease is an acute, subacute infection mucous membrane skin and the digestive system and sometimes different organs of birds, dogs, cats, calves, foal, pigs and humans[5]. However a commensal, *Candida albicans* is expected to inhabit the urogenital and gastrointestinal tract of a large percentage of the human population[6] It happens all over the world and risk factors in humans and pets can use of immuno suppressive drugs and chemotherapy in cancer patients, radiation therapy, the use of broad-spectrum antibiotic, new ways of life, the dense breeding animals and which causes this fungi is invasive[5,7] *Candida albicans* exhibits the ability to grow in a variety of reversible morphological forms [yeast forms (Y), pseudohyphal forms, and hyphal forms (H)] in response to various environmental signals[8] .The ability of *C. albicans* to switch its mode of growth has been shown to be required for the pathogenicity of this fungus[9] . Hyphae formation from yeast cells is a virulence trait enabling this fungus to invade host tissues [10]. While the use of new antifungal drugs, improve process control to fungal infection Tthe main problem still remains in the rapid diagnosis of infection . So start timely treatment is an important criterion in reducing the mortality rate of patients with immune suppression.[11] Detection of Candida species by traditional methods, including morphological and absorption test that is time consuming, also, when using automatic biochemical systems are sometimes misidentified clinical yeast isolates. Molecular detection methods based on the isolation and amplification Nucleic acids is include reaction-PCR Polymerase Chain and Isothermal amplification [12,13] Molecular methods have many advantages ,but each of the method have its own problems, for example, the PCR methods has ever can not Used publicly in all diagnostic centers due to the use of advanced equipment such as thermocycler[14,15]. loop-mediated isothermal amplification method (LAMP) is another method of isothermal amplification, Which is done amplification in conditions isothermal and therefore do not require a thermocycler, In addition, is

very high sensitivity and Specificity [16,11] .In reaction LAMP uses 4-6 primers recognizing 6-8 distinct regions of target DNA. A strand-displacing DNA polymerase initiates synthesis and 2 of the primers form loop structures to facilitate subsequent rounds of amplification. LAMP is rapid, sensitive, and amplification is so extensive that the magnesium pyrophosphate produced during the reaction can be seen by eye, making LAMP well-suited for field diagnostics. The amount of material and the temperature used of the LAMP test is given in the tables below [13,17] . Molecular methods Provides rapid and more accurate detection compared to traditional phenotypic methods. The purpose of this study is the rapid detection molecule with LAMP method for determine the presence of *Candida albicans* in animal samples which finally resulted in a rapid and accurate diagnostic method, and. can be done in all of the diagnostic centers for early detection with low cost of Candida albicans.

MATERIALS AND METHODS

Within a few months in one study of patients animal referred to a veterinary Clinicin Tehran that showed symptoms of Presence Candida albicans,20 samples who were randomly selected . Samples were collected in sterile tubes and were transported to the laboratory as soon as possible.

Preparation strains OF *Candida albicans* and culturing methods:

First, lyophilized and standard strains of Candida albicans belonging to Iranian Industrial Bacteria and Fungi Collection (Persian Type Culture Collection, PTCC) were cultured at GYEP liquid medium.

After the organism growth, 500 µl of the liquid medium was removed and centrifuged at 12000 rpm for 5 min. The supernatant was discarded and resulting precipitation was deionized in 100 µl of sterile double-distilled water and suspended; then, its DNA was extracted using phenol-chloroform methods[18] .

DNA extraction

Extracting DNA from standard strain: Phenol-chloroform method was used for DNA extraction [18].

100 µl of the cultured strain in the liquid medium was removed; then, at the first stage, 500 µl of the lysis solution or buffer lysis (Proteinase K= 250 µg/ml, Tris-Hcl=50 Mm, SDS=%10) was added.

At the second stage, 10 µl protease was added and shaken for 10 sec.

At the third stage, it was put in a 65° heater block for 20 min. After removal of the solution inside the tube, phenol-chloroform solution was added and, after 10 times of inversion, it was centrifuged for 5 min.

Afterward, the tube was placed on ice for 30 min. After removing from ice, the supernatant was transferred to a new tube and some isopropanol with the same volume of the solution was added to the tube. After 10 times of inversion, it was put in a -20° C freezer for 10 min. Once taken out of the freezer, it was centrifuged for 10 min at 12000 rpm and the supernatant was discarded; because isopropanol always causes DNA precipitation, the supernatant lacks any DNA. Afterwards, 1000 µl of alcohol 70% was poured on it and, after 10 times of inversion, centrifuged for 10 min at 12000 rpm (alcohol separates isopropanol from DNA). Finally, the supernatant was decanted (discarded), the test tube was placed in the 65° heater block, and 100 µl distilled water was added.

Primer design: LAMP primers using Primer explorer V4 software was designed for gene alph-INT1. Specific primers of *Candida albicans* are as shown in Table 1**.**

LAMP reaction:

The LAMP reaction was made in 25 µl by mixing 0.2 µM F3/B3, 1.6 µM FIP/BIP, 20 Mm Tris-Hcl, 10 Mm Kcl , 10 mM (NH4)2SO4 , 9 mM MgSO4, 1.4 mM dNTP, 0.8 M Betain (Sigma- Aldrich), 8 u Bsm DNA polymerase (New England Biolabs). Themixture was incubated at 61 for 1 h.

Table 1: LAMP primers were designed for α INT1

Gene	Primer name	Sequence (5' → 3')
alph-INT1	F3	CAATGGAAAGATCCTTCTCAA
	B3	TGTTATCTCTCTTGTGTGTCAT
	FIP	AGGTTTCGTCGTATGAAGTGGT-ATTCTGATGAAGATACAAATGCT
	BIP	CAACGAAGTCAATCTGGAACCA-AAATTGCTGAAATTTTCGCG

Table 2: The materials used in the LAMP test

materials used in the LAMP test	
10X LAMP Buffer	2.5 µl
Betaine	4 µl
dNTP(10mM)	3 µl
Primer Master Mix	1 µl
$MgSo_4$(100mM)	1.8 µl
BSM DNA Pol 8U/ µl	1 µl
D.D.W	6.7 µl
Template DNA	5 µl

Determine the limit of detection LOD and specificity of the LAMP test: In order to determine the LOD of these techniques in this research, the standard strain of *Candida albicans* was applied in preparing a series of dilutions from 10 million copies to five copies of DNA to LAMP reactions.

Test specificity

DNA from different microorganisms such as *Cryptococcus neoformance, Fusarium spp* **,** *Fusarium Solani, Aspergillus parasiticus, Escherichia coli (E.coli), Hepatitis Bvirus (HBV), Aspergillus flavus* with the DNA of the fungus *Candida albicans* and specific primers for fungus

placed in tube different and was performed LAMP test. Test specificity should be done separately the fungus *Candida albicans*.

And also DNA from different microorganisms such a *Candida parapsilosis, Candida dubliniensis, Candida glabrata and Candida tropicalis* and specific primers for fungus placed in tube different and was performed LAMP test**.**

RESULTS

Optimized LAMP product with DNA extracted from standard strains The reaction by adding SYBR-Green were observed Positive tube bright green and negative tube very low orange colors (Figure 1).

LOD test

LOD of LAMP test with specific primers for *Candida albicans* could identify at least 10 copies of DNA related to each fungus of *Candida albicans*

Specificity test

DNA from different microorganisms such as *Cryptococcus neoformance, Fusarium spp, Fusarium Solani, Aspergillus parasiticus, Escherichia Coli (E.Coli), Hepatitis Bvirus(HBV),* human DNA with the DNA of the fungus *Candida albicans* and specific primers for fungus placed in tube different and was performed LAMP test

LAMP test results

On 20 samples from animals patient tested, 19 were positive for Candida albicans (Figure 4).

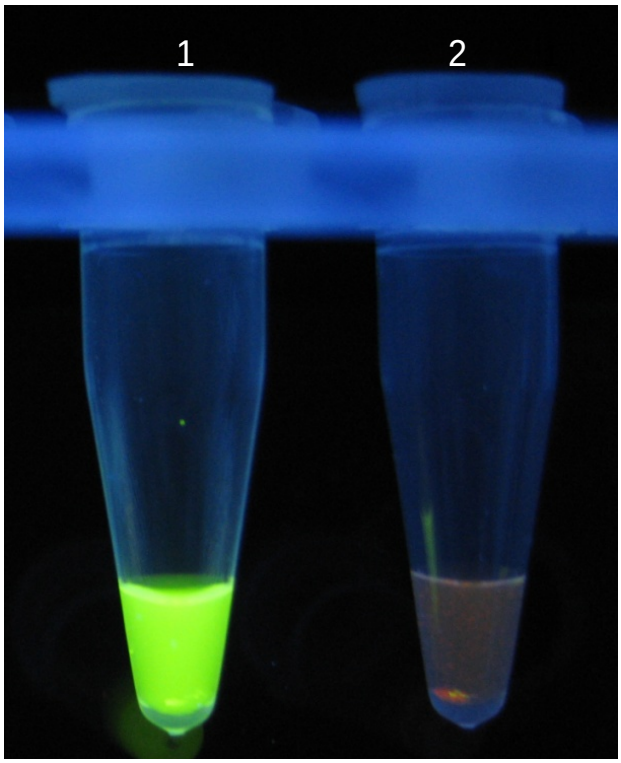

Figure 1: LAMP Optimized Test
Tube 1: gene-specific amplification of *Candida albicans;* Tube 2: negative control

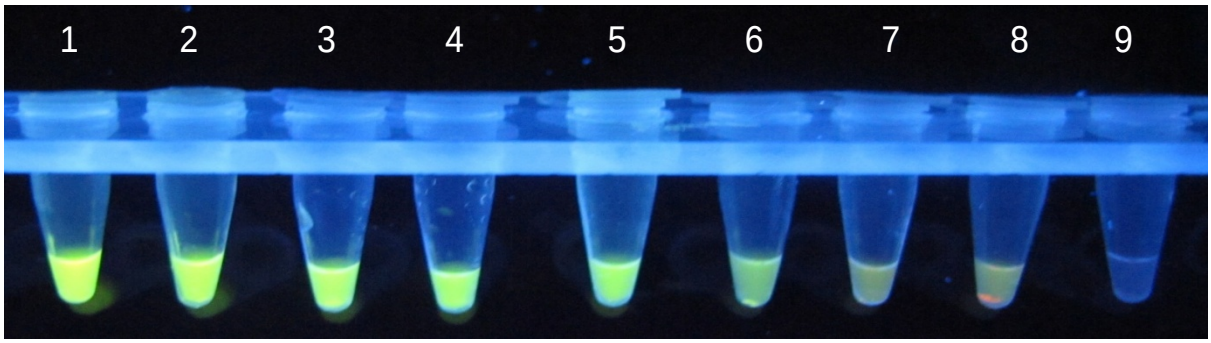

Figure 2: Results of LOD test
Tube 1: Positive control; Tube 2: 1,000,000 copies of DNA, *Candida albicans;* Tube 3: 100000 copies of DNA, *Candida albicans;* Tube 4:10000 copies of DNA, *Candida albicans;* Tube 5:1000 copies of DNA , *Candida albicans;* Tube 6: 100 copies of DNA , *Candida albicans;* Tube 7:10 copies of DNA , *Candida albicans;* Tube 8: 1 copy of DNA , *Candida albicans;* Tube 9: Negative control

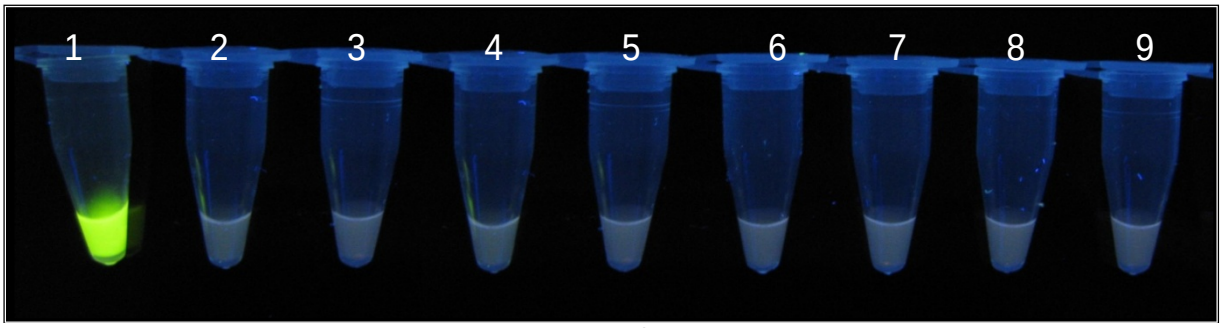

Figure 3A: Specificity test
Tube1: DNA of *Candida albicans;* Tube 2 to 8 and: DNA *Cryptococcus neoformance* , *Fusarium spp, Fusarium solani,* Aspergillus parasiticus, Aspergillus *flavus* , *Escherichia Coli* (*E.Coli*), *Hepatitis Bvirus*(HBV); Tube 8: negative control; Primers designed reacted with *Candida dubliniensis* but no reaction with other Candida species

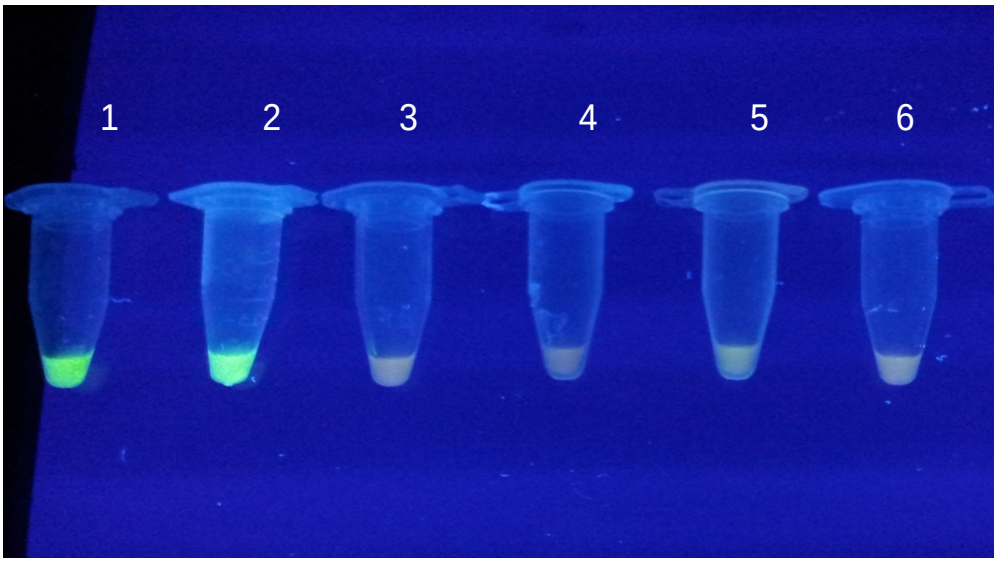

Figure 3B: Specificity test
Tube1: DNA of *Candida albicans;* Tube 2: DNA *Candida dubliniensis;* Tube 3, 4, 5: Candida *parapsilosis* , *Candida tropicalis* , *Candida glabrata;*Tube6: negative control

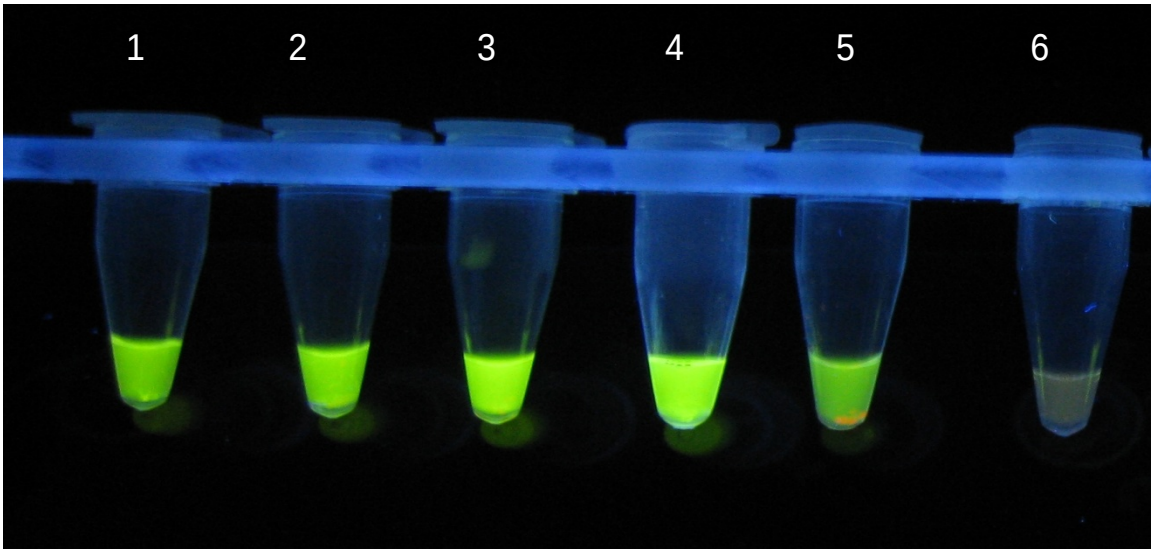

Figure 4: Results of LAMP test on animal samples
Tube 1: Positive control; Tube 2,3,4,5 : positive samples of *Candida albicans;* Tube 6: negative control

DISCUSSION AND CONCLUSIONS

Opportunistic infections caused by yeasts in recent decades has been of great importance [19]. Invasive candidiasis is a common and potentially fatal side effects caused by treatment, especially chemotherapy, cancer patients Candida species are the most common cause of yeast infections in animal [5] . Also Is true Incidence of candidiasis in humans , so that candidiasis fungal is included 66-80% of fungal infections . The incidence of disseminated candidiasis in 1990 was approximately 80% of all cases of candidaemia due to *Candida albicans.* Recent reports show from other countries, a change in the epidemiology of candidiasis [20] . This study primer the desired is designed from sequences encoding gene alph-INT1 *Candida albicans* . This gene is similar to vertebrates leukocytes eyntgren [21] . this primer In terms of Specificity with *Candida spp , Cryptococcus neoformance, Fusarium spp , Fusarium Solani, Aspergillus parasiticus, Aspergillus flavus, E.ColiEscherichia Coli, Hepatitis Bvirus(HBV)* were tested , and just alph-INT1 attached and doing Proliferation, but this primers was reacted with *Candida dubliniensis* . LAPM method is very simple of gene amplification method that given temperature of beginning to the end. LAMP New novel technique is one of the very simple gene amplification methods which were performed under isothermal condition. In contrary with its simplicity it has high specificity and sensitivity with obviating the need for using thermal cycler [17 ,22]. LAMP method is used for detection, identification and isolation candidiasis of other fungi This method is simple and also has very high sensitivity and Specificity. And also is performed at low cost Because it does not need to device and hardware like thermocycler[17] . The reaction is administered external primers

(F3, B3) and internal primers (FIP, BIP) which each one recognizes 2 separate distinct site and also two special loop primers which recognize a total of 8 distinct sequences in the DNA target. The amplification products are stem-loop DNA structure with several inverted repeats and cauliflower- like structure with multiple loops [14,23,24]. This method has many advantages and The method is amplification DNA at a certain temperature and in a short time And can be started diagnosis with a few DNA Reason High Sensitivity in this method is highly specific Amplification of target genes because is able to with 4 primer at the beginning of the reaction have been identified 6 region sequences of the target gene and during the reaction 4 region. This technique does not require advanced facilities and the reaction is done by using a simple Dry Plate Reaction verification was with the addition of cyber-lamp and observed under UV light easily without having to electrophoresis in a very short time. Molecular methods have helped to rapid and accurate detect of Candida albicans Rapid and available detection *Candida albicans* and timely treatment of patients be prevented from rising costs patients the results of this study are help to remove disease and avoid increasing costs to patients for early diagnosis. Therefore LAMP technique is a rapid, sensitive and specific method and not needing to improved instruments higher sensitivity other molecular methods and could be a proper replacement for it in future. and also low cost for diagnosis of systemic candidiasis in samples such as pets in laboratory centers

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