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## Original article

# Loop-Mediated Isothermal Amplification: A rapid and simple method for detection of fluconazole resistant *Candida albicans* vaginitis

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## ABSTRACT

**Background:** Candidiasis is one of the most important opportunistic fungal infections. Time-saving antimicrobial susceptibility testing (AST) and molecular methods were developed to detect antimicrobial resistance. Loop-mediated isothermal amplification (LAMP) is a molecular diagnostic method performed isothermally with Bst DNA polymerase and four or six primers. **Objective:** Vitek identification of *Candida* isolates and determination of their AST were performed. However, it is time consuming; therefore, rapid and sensitive LAMP-based assay was tested as a method for phenotypic detection of resistance of *Candida albicans* isolates to fluconazole. **Methodology:** Identification of *Candida spp.* isolated from vaginal swabs of non-pregnant women with vaginitis and minimal inhibitory concentration (MIC) values were done by Vitek2 system. Sensitive *C. albicans* isolates were inoculated into sabouraud's dextrose broth without fluconazole while resistant strains were inoculated into broth with fluconazole and incubated at 37°C. At end of 2nd, 4th and 6th h, cultures were harvested by 10 min centrifugation at 2,000 rpm and DNA was extracted by boiling. For LAMP, specific primers of *C. albicans* alpha-INT1 gene were used. **Results:** Out of 250 samples, 117 (46.8 %) were positive for *Candida* infections. *Candida albicans* isolates (57 isolates) were grouped into fluconazole-sensitive (12 isolates) and fluconazole-resistant (45 isolates). Fluconazole-resistant *C. albicans* could grow in presence of drug and positive LAMP reaction was obtained after a time interval similar to sensitive isolates. **Conclusion:** LAMP reaction allowed phenotypic detection of behavior of resistant strains in presence of antifungal agent. LAMP based evaluation of AST is superior to conventional methods and molecular detection of resistance genes.

## Introduction

Candidiasis is one of the most important opportunistic fungal infections that affect the skin, nails and mucosal surfaces (vaginal, bronchial, digestive...etc.). Sometimes, the disseminated

infection may also occur in kidneys, liver and heart. In immune-compromised patients, *Candida spp.* are among the main causes of sepsis and bloodstream infections that have been associated with significant mortality [1].

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The accurate detection of *Candida spp.* has a significant role in the prevention, control and treatment of the disease. The routine laboratory diagnosis of *Candida spp.* is carried out by using diagnostic methods such as CHROMagar *Candida*, germ tube production, chlamydoconidia generation and carbohydrate assimilation assays [2].

The fatalities caused by resistant pathogens' infections are increasing every year. Therefore, administration of adequate antimicrobial agent against the target microbe in the treatment of infectious diseases as well as prompt antimicrobial susceptibility testing (AST) in a microbiology laboratory are essential [3]. However, conventional AST in a routine laboratory setting is time consuming [4].

Recently, time-saving AST methods have been developed to detect antimicrobial resistance by detecting particular resistance genes using molecular approaches [5].

The loop-mediated isothermal amplification (LAMP) assay is a molecular diagnostic method performed under isothermal conditions with *Bst* DNA polymerase and four or six independent primers that recognize at least six distinct sequences on the target DNA [6] with possible real-time measurement of the LAMP reaction because DNA amplification is directly correlated with the turbidity derived from magnesium pyrophosphate (i.e. results can be visually distinguished by the naked eye on account of the production of a large amount of magnesium pyrophosphate precipitation) [7]. Because LAMP technique is simple, sensitive and cost effective, LAMP-based assay was tested in this work as a method for phenotypic detection of resistance of *Candida albicans* (*C. albicans*) isolates to fluconazole antifungal.

## Materials and Methods

### Ethics approval

Human Ethics Committee of Faculty of Medicine in Assiut University approved this study. IRB local approval number: 17300590. We obtained informed consent before collecting samples from all patients.

**Study design:** Cross sectional study.

**Subjects:** This study included 250 non-pregnant women in the reproductive age with clinical manifestations of vaginal infection attended to the outpatient gynecology clinic at Women's Health Hospital, Assiut University from May to October; 2021. Vaginal swabs were obtained from eligible

participants. Patients who received antifungal treatment within three days before taking the swab were excluded from the study.

**Flow of specimen processing:** The study was designed as shown in **figure (1)**.

1. A single separate colony of fluconazole sensitive *C. albicans* isolate was inoculated into drug free sabouraud's dextrose broth (used as a control). The time needed to detect positive LAMP reaction was expressed as threshold time (Tt).
2. Tt is inversely proportional to the growth rate of *C. albicans* (i.e. with more *C. albicans* growth, the harvested cells from the broth culture are more with enhanced opportunity to get positive LAMP reaction).
3. A single separate colony of *C. albicans* isolate assessed for fluconazole resistance was inoculated into sabouraud's dextrose broth supplemented with fluconazole at a value equals to the minimal inhibitory concentration (MIC) break point of sensitive isolates (according to the CLSI, 2020).
4. If this isolate is resistant to fluconazole, growth of fungal cells will occur in the presence of the drug and the time needed to get positive LAMP is expressed as Ttd.
5. The threshold time needed to get positive LAMP for fluconazole free broth culture (Tt) was subtracted from threshold time needed to get positive LAMP for fluconazole supplemented broth culture (Ttd) and the time was expressed as delta threshold time (DTt). The lower the DTt, the more the resistance expressed by *C. albicans* isolate (the isolate can grow in the presence of fluconazole at a rate similar to its ability for growth in its absence).

### Culture on sabouraud's dextrose agar (SDA) plates:

Vaginal swabs were inoculated into SDA plates (Himedia, India) supplemented with chloramphenicol (50 mg/L) and incubated at 37°C for 48 h. Yeast growth was confirmed by microscopic examination of Gram stained smear prepared from obtained colonies.

**Identification of *Candida spp.* and determination of their susceptibility pattern by Vitek 2 automated yeast identification system (bioMérieux, France):**

Different *Candida spp.* obtained on SDA plates were sent to South Egypt Cancer Institute Microbiology laboratory, Assiut to be identified by YST cards and their MIC values were determined by ASTYS08 cards. MIC break points were interpreted according to CLSI, 2020.

As *C. albicans* were the most common cause of Candidiasis, they were selected to be tested by LAMP based antifungal susceptibility testing (AFST).LAMP based AFST was performed by using fluconazole as it is the most commonly prescribed drug for treatment of Candidiasis [8].

**Time needed to obtain positive LAMP reaction for *C. albicans* isolates:**

Sensitive *C. albicans* isolates(used as a control) were inoculated into sabouraud’s dextrose broth (Himedia, India) and resistant strains were inoculated into sabouraud’s dextrose broth to which

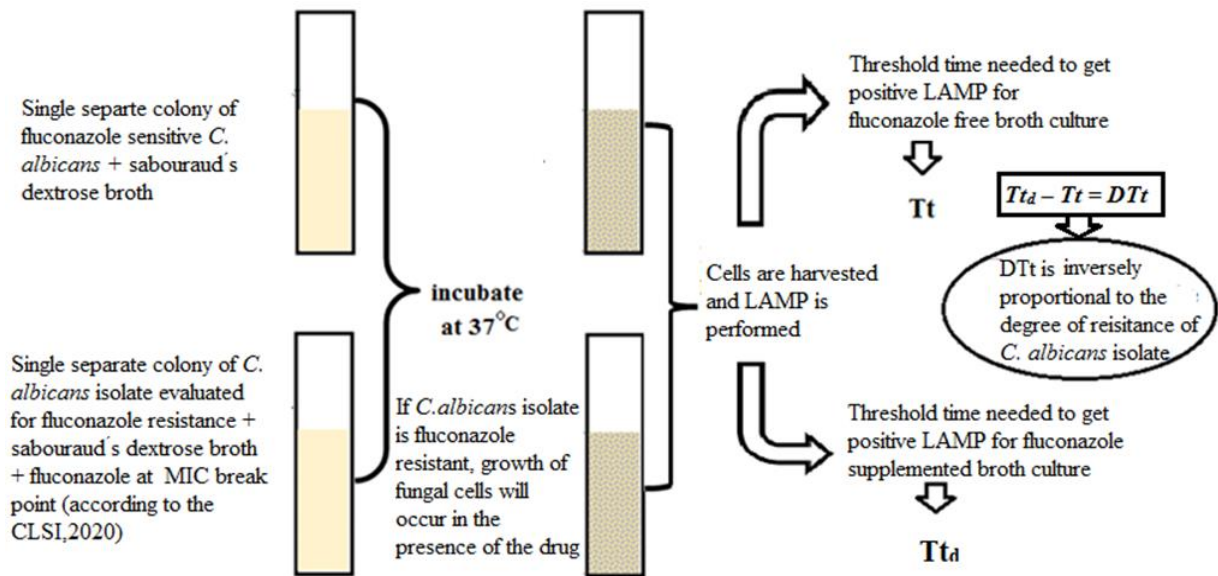
fluconazole (Bio-Rad, Egypt) was added at a concentration equals to the MIC of sensitive isolates (according CLSI, 2020)then tubes were incubated at 37°C. At the end of second, fourth and sixth hours, the cultures were harvested by centrifugation for 10 min at 2,000 rpm. We used normal saline to wash the cells and DNA extraction was carried out by boiling method [9].

To perform LAMP reaction, specific primers of *Candida albicans* alpha-INT1 gene were used (Table 1).

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 (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 9 mM MgSO<sub>4</sub>, 1.4 mM dNTP, 0.8 M Betaine (Sigma- Aldrich), 8 u *Bst* DNA polymerase (New England Biolabs). The mixture was incubated at 61°Cfor 1 h. The results of LAMP reaction were visually recognizable by agarose gel electrophoresis to detect the characteristic ladder appearance of positive reaction.

$$T_{td} - T_t = DTt$$

So comparable  $T_t$  and  $T_{td}$  with  $DTt$  approaching zero value can be used as a fast, simple and cost-saving method to detect drug resistance among clinical *C. albicans* isolates.



**Table 1.** The nucleotide sequence of primers targeting the alpha-INT1 gene of *Candida albicans* used in the LAMP reaction [10].

<b>F3</b>	5'-CAATGGAAAGATCCTTCTCAA-3'
<b>B3</b>	5'-TGTTATCTCTCTTGTGTGCAT-3'
<b>FIP</b>	5'-AGGTTTCGTCGTATGAAGTGGT-ATTCTGATGAAGATACAAATGCT-3'
<b>BIP</b>	5'-CAACGAAGTCAATCTGGAACCA-AAATTGCTGAAATTTTCGCG-3'

## Results

Out of the 250 sample included in the study, 117 (46.8 %) were positive for *Candida* infection. The types of *Candida* isolates as identified using Vitek 2 automated yeast identification system (YST cards) are listed in **table (2)**.

Antifungal susceptibility testing was performed for all *Candida* isolates using Vitek 2 automated yeast identification system (ASTYS08 cards) and the results are listed in **table (3)**.

According to sensitivity to fluconazole, *C. albicans* isolates (57 isolates) were grouped into two groups: fluconazole sensitive group (12 isolates) and fluconazole resistant group (45 isolates). The sensitive group was used as a control group to determine the threshold time needed to detect positive LAMP reaction (Tt) after incubation into drug free sabouraud's dextrose broth. The

fluconazole resistant group was tested to determine the threshold time needed to detect positive LAMP reaction after incubation into sabouraud's dextrose broth to which fluconazole was added (Ttd) at the end of 2<sup>nd</sup>, 3<sup>rd</sup> and 4<sup>th</sup> h from which DTt can be calculated (**Figure 2 and Table 4**).

From **table (4)**, it is obvious that fluconazole resistant strains of *C. albicans* could grow even in the presence of the drug so DTt values were zero for most of these resistant strains. For some of the resistant strains DTt values were comparable to sensitive strains (2 h). Therefore, LAMP reaction allowed phenotypic detection of behavior of resistant strains in the presence of antifungal agent i.e. DTt is inversely proportional with isolate MIC.

**Table 2.** Types of 117 (46.8%) *Candida* isolates as identified by Vitek 2 automated yeast identification system (YST cards).

Type of isolate	No. (Percent of total positive)	Type of isolate	No. (Percent of total positive)
<i>C. albicans</i>	57 (48.72%)	<i>C. haemulonii</i>	3 (2.56%)
<i>C. krusi</i>	3 (2.56%)	<i>C. inconspicua</i>	3 (2.56%)
<i>C. glabrata</i>	9 (7.7%)	<i>C. dubliniensis</i>	6 (5.13%)
<i>C. tropicalis</i>	15 (12.82%)	<i>C. ciferrii</i>	18 (15.38%)
<i>C. rugosa</i>	3 (2.56%)		

**Table 3.** Antifungal susceptibility testing of *Candida* isolates as measured by Vitek 2 automated yeast identification system (YST cards).

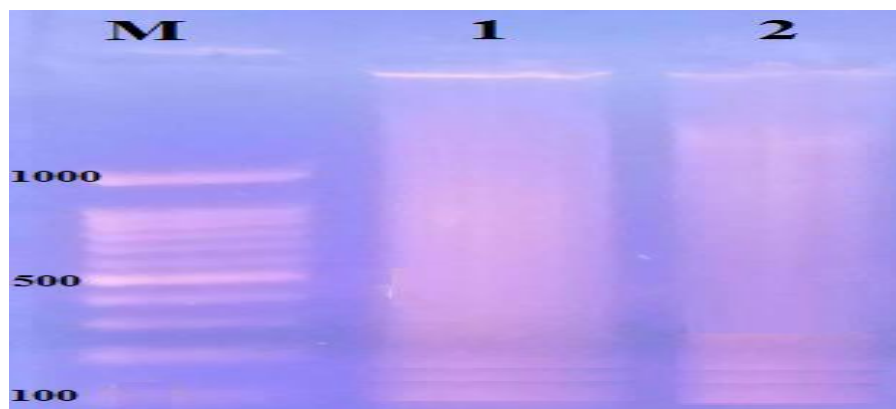
	Fluconazole			Voriconazole			Capsfungin			Micafungin			Amphotericin B			Flucytosine		
	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R
<i>C. albicans</i> (57)	12	0	45	57	0	0	54	0	2	57	0	0	57	0	0	57	0	0
<i>C. krusi</i> (3)	-	-	-	3	0	0	3	0	0	3	0	0	3	0	0	0	0	3
<i>C. glabrata</i> (9)	-	-	-	9	0	0	9	0	0	9	0	0	9	0	0	6	3	0
<i>C. tropicalis</i> (15)	9	0	6	15	0	0	15	0	0	15	0	0	15	0	0	15	0	0
<i>C. rugosa</i> (3)	-	-	-	3	0	0	-	-	-	-	-	-	3	0	0	3	0	0
<i>C. haemulonii</i> (3)	-	-	-	3	0	0	-	-	-	-	-	-	3	0	0	3	0	0
<i>C. inconspicua</i> (3)	-	-	-	3	0	0	-	-	-	-	-	-	3	0	0	3	0	0
<i>C. dubliniensis</i> (6)	-	-	-	6	0	0	-	-	-	-	-	-	6	0	0	3	3	0
<i>C. ciferrii</i> (18)	-	-	-	18	0	0	-	-	-	-	-	-	18	0	0	-	-	-

S: sensitive, I: intermediate sensitive, R: resistant

**Table 4.** Time needed to detect positive LAMP reaction and its relation to fluconazole MIC values of *C. albicans* isolates.

<i>C. albicans</i> sensitivity to Fluconazole			Time needed to detect positive LAMP reaction		
No.(Percent of total <i>Candida</i> isolates)	Results	MIC	No. of isolates at the 2 <sup>nd</sup> h	No. of isolates at the 4 <sup>th</sup> h	No. of isolates at the 6 <sup>th</sup> h
12 (21.1%)	S	≤ 0.5	12 (Tt)	0	0
6 (10.5%)	R	8	0	6 (DTt = 2 h)	0
39 (68.4%)	R	16	26 (DTt = 0)	13 (DTt = 2 h)	0

**Figure 2.** Agarose gel electrophoresis of LAMP amplified alpha-INT1 gene of fluconazole sensitive (1) and fluconazole resistant (2) *C. albicans* (The positive reaction is manifested as a ladder-like pattern on the 1.5% agarose gel due to the mechanism of LAMP reaction as a loop amplifier that uses at least 4 primers). M is 100 bp DNA ladder marker.



**Discussion**

The conventional methods for antifungal susceptibility testing are time consuming which is not suitable in cases of life threatening infections.

Molecular diagnostic methods (as PCR) that detect resistance genes require expensive equipment and not available in all laboratories. In addition, these

methods do not allow detection of gene behavior (whether expressed or not), so it is not possible to predict the phenotypic antifungal susceptibility pattern. These factors increased the demand for more time-saving and accurate methods of antifungal susceptibility testing.

Results of the present study concluded that the percent of vaginal *Candida* infection was 46.8 % of all participants in the study and the most frequent *Candida* isolate was *C. albicans* (48.72% of total positive samples). These results are matched with **Anh et al.** [11] study who declared that vaginal yeast colonization in non-pregnant women was 51.3% of participants and *C. albicans* was the most frequent (51.37%). As **Taei et al.** [12] study declared that the increasing of non-*albicans* *Candida* (NAC) species have been recognized significantly during the last two decades, results of the present study showed that the NAC represented 51.28% of total positive samples. This represents a major problem that calls for more studies to standardize their laboratory identification methods and to set up susceptibility break points of antifungal drugs against these new infecting pathogens.

Results of the present study showed that 78.9% of *C. albicans* isolates were resistant to fluconazole that is consistent with **Berkow and Lockhart** [13] who concluded that in the past two decades, several genes and mutations that increase resistance to fluconazole in clinical isolates, primarily in *C. albicans*, have been elucidated.

According to **Knoll et al.** [14], the recent rise in the drug resistance of fungal pathogens, AFST is becoming increasingly relevant. As the common methods of AFST have turnaround times of 24 to 48 h, new rapid methods to detect resistances are necessary in order to ensure the fast and adequate adaption of antifungal therapy when needed. **Ota et al.** [15] applied the LAMP technique to detect antimicrobial resistance in clinical *Escherichia coli* isolates. They cultivated targeted bacteria for a short period with and without antibiotic before the LAMP reaction. The time needed to detect a positive reaction with LAMP was used to generate a threshold time (Tt) value, and subtraction of the Tt value for an antibiotic-free sample from the Tt value in an antibiotic-exposed sample generated the DTt value, which was used as a marker of antimicrobial susceptibility. The present study applied the same principle to establish rapid, cost-saving and accurate method to detect fluconazole resistance in *C. albicans* clinical

isolates. We found that the time needed to get positive LAMP reaction for fluconazole resistant strains cultured in the presence of the drug is comparable to the time needed for sensitive isolates grown in drug free medium (Tt) so DTt is nearly zero that is matched with **Ota et al.** [15] who declared that the DTt value can be used as a marker of microbial susceptibility as the time needed to get positive LAMP reaction of resistant strains was unchanged, despite antibiotic exposure.

## Conclusions

Loop-Mediated Isothermal Amplification based evaluation of antifungal susceptibility is superior to both conventional methods and molecular detection of resistance genes. For resistant isolates, the time needed to get positive LAMP reaction in the presence of the antifungal drug is comparable to the time needed to get positive LAMP reaction for sensitive control strains in the drug free tubes (i.e. the lower the DTt value the more the resistance of the strain to the tested antifungal agent). Future studies are required to validate Tt (threshold time) breakpoints needed to get positive LAMP reaction in correlation to the degree of resistance of fungal isolates for its use in clinical practice.

## Limitations of the study

The major limitation of this study is the reading of the final result of LAMP reaction gives a qualitative result. Other limitations include that this study has to be standardized for the remaining antifungal agents (other than fluconazole) and repeated for other fungal species (other than *C. albicans*). Proper calculation of the breakpoints of Tt for each fungal strain against each antifungal agent is mandatory. In addition, it is suggested to apply the same research protocol directly for clinical samples with suspected fungal pathogens to evaluate its effectiveness for early determination of antifungal susceptibility pattern.

## Conflict of interest

The authors have no conflicts of interest to declare that are relevant to the content of this article.

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## Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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