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ORIGINAL PAPER

Diagnosis of *Candida albicans*: conventional diagnostic methods compared to the loop-mediated isothermal amplification (LAMP) assay

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Abstract

Candida species cause a wide range of opportunistic infections in humans and animals. The detection of *Candida* species by conventional diagnosis methods is costly and time consuming. This study was conducted for the first time to evaluate and compare a relatively new molecular assay and the loop-mediated isothermal amplification (LAMP) technique with conventional methods for detection of *Candida albicans*. In this study, 70 different species of *Candida* identified by conventional methods were cultured on Sabouraud chloramphenicol agar medium and then the genomic DNA was extracted. The LAMP technique was performed using specific primers targeting the ITS2 gene of *C. albicans*. The analytical sensitivity and specificity of LAMP were measured using a tenfold serial dilution prepared from extracted DNA from standard *C. albicans* strain from 1 ng to 1 fg and the DNA samples of other clinical *Candida* species and three non-*Candida* yeast. Out of 70 yeast samples analyzed by LAMP technique, 24 samples (34.3%) were positive for *C. albicans*. Comparison of the results showed that the CHROMagar *Candida* and germ tube production methods are quite consistent with the LAMP technique, while the agreement amount between the results of carbohydrate assimilation and chlamydoconidia generation assays and LAMP technique was 98.5% and 72.8%, respectively. The detection limits of the LAMP assay were 10 fg of the DNA from the standard *C. albicans* strain. No amplification was observed in the DNA samples of other yeast species and only the DNA sample of standard *C. albicans* strain was amplified. Based on the results, it can be concluded that the LAMP method is as specific and precise as common diagnostic methods, but is faster, easier deployable or more sensitive. Therefore, this method can be used as a suitable complementary assay for *Candida* diagnosis in medical diagnostic laboratories and field conditions.

Keywords *Candida albicans* · Diagnosis · Conventional diagnostic methods · LAMP technique

Introduction

Candidiasis is one of the most important opportunistic fungal infections which affect the skin, nail, vaginal, bronchial, lungs, and digestive mucosal surfaces acutely, subacute and chronically (Mirhandi et al. 2006). Sometimes, the disseminated infection may also occur in kidneys, liver, and heart (Mirhandi et al. 2006; Chadwick et al. 2013). In immunocompromised patients, the *Candida* species are among the main causes of sepsis, and nosocomial bloodstream infections which have been associated with significant mortality

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(Lion 2017). Also, *Candida* species can cause the infections of the prosthesis, the urinary tract, and upper respiratory tract. *Candida albicans* has an indisputable ability to develop human infections, as is the most common fungal infectious agent in humans, the most common fungal agent of hospital infections and the fourth cause of nosocomial bloodstream infections among all microbial agents that cause infection (Coronado-Castellote and Jiménez-Soriano 2013; Sherry et al. 2014). Increasing strains resistant to Azole compounds such as fluconazole which used in the prevention and treatment of candidiasis is one of the main reasons for increased *Candida* infections (Saravana Bhavan et al. 2010; Ruhnke et al. 2011). The virulence factors of *C. albicans* include polymorphism, secretory enzymes, growth potential at high temperatures and drug resistance (Katiraee et al. 2014).

The accurate detection of *Candida* species has a significant role in the prevention, control and timely treatment of the disease. The routine diagnosis of *Candida* species in the laboratory is carried out using diagnostic methods such as CHROMagar *Candida*, germ tube production, chlamydoconidia generation, and carbohydrate assimilation assays (Pincus et al. 2007). The CHROMagar *Candida* medium is one of the most commonly used culture media for the isolation of commonly *Candida* spp. strains. After growth on this medium, the colony of each *Candida* species is stained in particular color (Pincus et al. 2007; Lion 2017). This diagnosis method is cost effective and requires at least a short time to achieve the results. Also, this method cannot differentiate all *Candida* species, and due to the color similarity of the colonies, it is difficult to accurately differentiate the species (Pincus et al. 2007; Chadwick et al. 2013). Germ tube production is a preliminary test for the diagnosis of *C. albicans*, which takes 2–3 h. This method is under the influence of bacterial contamination and requires a specialist to perform the test. However, germ tube production is also seen with *C. dubliniensis* and *C. stellatoidea* (Anaissie et al. 2009). In the germ tube production method, as well as other conventional diagnostic methods, the amount of inoculated organism, type of protein liquid, and temperature are effective in the results of the test. Besides, the germ tube production method is not accurate and has false-negative results (Kim et al. 2002; Pincus et al. 2007). Chlamydoconidization requires the culture to be carried out in a Corn Meal Agar. In this semi-starvation medium, *C. albicans* produces chlamydoconidia after 4 days (Pincus et al. 2007). The commercial kits are based on the carbohydrate assimilation assays which a rapid diagnosis can be achieved using this method. This test is not effective lonely, and the chlamydoconidization method should always be used alongside it (Mirhandi et al. 2006; Pincus et al. 2007). Identifying some unusual species with these methods is difficult and sometimes impossible. Since the rapid and accurate identification of *C. albicans* from the non-*albicans* species is essential, especially in people who have a recurrent infection (Anaissie et al. 2009), medical diagnostic laboratories need to increase their ability to quickly diagnose these yeasts by relying on simple, valid and inexpensive methods with high sensitivity and specificity.

Molecular detection methods based on the isolation and amplification of nucleic acids of the fungus include a variety of polymerase chain reaction (PCR) assays, hybridization, and loop-mediated isothermal amplification (LAMP) technique, which can detect the infection even in the primary stages of the disease (Inácio et al. 2008). The PCR assay is a fast and reliable method that has not only many advantages but also some limitations including various temperature cycles, the use of expensive thermocyclers and gel documentation system, and variable sensitivity and specificity. Therefore, this method cannot be used in low income countries or in field conditions (Zhi-xiang et al. 2010). The relatively new DNA amplification method, LAMP technique, is a simple, rapid, sensitive and cost-effective method with high performance that does not have the above-mentioned limitations. In this method, the DNA is specifically and efficiently amplified under isothermal (63–67 °C) conditions (Inácio et al. 2008; Rostami et al. 2018). Due to the use of the *Bst* DNA polymerase enzyme with strand displacement activity, this technique does not require thermocyclers and the test results are detectable with the naked eye by adding SYBR Green I or other DNA intercalating fluorescent dye to the reaction tubes at the end of the reaction. Therefore, there is no need for electrophoresis of the products on the agarose gel and, generally, it is suitable for the limited conditions (Chen and Ge 2010; Reddy et al. 2010).

This study was conducted for the first time to evaluation and comparison of the LAMP technique with common diagnostic methods such as CHROMagar *Candida*, germ tube production, chlamydoconidia generation, and commercially available carbohydrate assimilation assays for rapid and sensitive detection of *C. albicans*.

Materials and methods

Study population and clinical samples

In this study, 70 yeast samples which were collected from 70 patients with clinical symptoms, the different species of *Candida* identified by conventional methods, including CHROMagar *Candida*, germ tube production, chlamydoconidia generation, and ID 32C kit have been used. The standard strains of *C. albicans* Persian Type Culture Collection 5027 (PTCC, CW Emmons, IRAN) and *Candida glabrata* (*C. glabrata*) ATCC 2001/CBS 138 (ATCC/CBS, JCM, IRAN) which were used as positive and negative controls, respectively, were kindly repapered from the Department

of Medical Mycology, Iran University of Medical Sciences, Tehran, Iran.

From the suspension of each yeast sample, 0.2 ml was stored in the refrigerator within sterile distilled water. After vortex and homogenizing, the suspensions were transferred onto a Sabouraud chloramphenicol agar medium under the sterile conditions and then incubated at 35 °C for 24 h. The DNA from all yeasts cultured was extracted using the DNA extraction kit (DNG-PLUS, Sinnagene, Iran) according to the manufacture's protocol. Electrophoresis and a Nano spectrophotometer (WPA-Biowave II, England) were used to determine the purity (A260/230, A260/280 ratios) and quality (ng/uL) of DNA.

LAMP reaction

Five oligonucleotide primers targeting seven conserved regions within the sequence of internal transcribed spacer 2 (ITS2) gene of *C. albicans* were used for the LAMP assay (Table 1) (Kasahara et al. 2014). The LAMP reaction mixture (25 µL) contained 1 µl of template DNA (100 ng/µL), 40 pmol each of primers FIP (Forward Internal Primer) and BIP (Backward Internal Primer), 20 pmol of primer LB (Loop Backward), 5 pmol each of the primers F3 (Forward 3) and B3 (Backward 3), 8 U of *Bst2* DNA polymerase (New England Biolabs, Ipswich, MA, USA), 2 × reaction buffer (40 mM Tris–HCl (pH 8.8), 20 mM KCl, 20 mM $(NH4)_2SO_4$, 16 mM $MgSO_4$, and 0.2% Tween 20), 0.8 M betaine (Sigma-Aldrich), and 1.4 mM deoxynucleoside triphosphates (dNTP). Because the LF primer was not designed for the ITS2 gene of *C. albicans*, the twice-distilled water was used instead of the LF primer in the LAMP reaction.

The volume of reagents was calculated according to the total number of samples and prepared as a master mix, then 24 µl of this mixture was poured into the new microtubes separately, and thereafter 1 µl of the DNA of each sample was added to each microtubule. The extracted DNA from standard strains of *C. albicans* (PTCC 5027) and *C. glabrata* (CBS 138) was used as positive and negative controls, respectively. The reaction mixture was incubated in a water bath at 62 °C for 60 min and was inactivated at 80 °C for 2 min. The resulting amplicons were detected by visual observation after addition of SYBR Green I (Invitrogen Carlsbad, CA, USA) to the reaction microtubes. The optimum time and temperature conditions for the LAMP assay were determined by carrying out the reactions at 62–67 °C for 30, 45, 60 and 75 min in duplicate.

Table 1 The nucleotide sequence of primers targeting the ITS2 gene of *Candida albicans* used in the LAMP reaction

Target yeast	Primer set	Primer Sequence (5′-3′)
Candida albicans	F3	TCTGGTATTCCGGAGGGC
	B3	AGTCCTACCTGATTTGAGGT
	FIP	CTACCGTCTTTCAAGCAA ACCCATGAGCGTCGTTTC TCCCT
	BIP	TTGACAATGGCTTAGGTC TAACCAAAAGATATACGT GGTGGACGTTAC
	LB	CTCAACACCAAACCCAGCGG

Confirmation of the LAMP primer specificity

To confirm the specificity of the LAMP primers for the detection of *C. albicans*, a PCR assay was performed with the two F3 and B3 external primers (Table 1). The PCR mixtures (20 µL) consist of 0.1 U of *Taq* DNA polymerase, 5 µmol/L of each of the F3 and B3 primers, 10 mmol/L of Tris–HCl, pH 8.3 (at 25 °C), 50 mmol/L of KCl, 1.5 mmol/L of $MgCl_2$, 250 µmol/L of each dNTP, and l µL of extracted DNA (100 ng/µL). The PCR assay was performed with an initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation (30 s at 94 °C), annealing (30 s at 52 °C), and extension (30 s at 72 °C), with a final extension for 5 min at 72 °C. Ten microliters of the PCR products was subjected to electrophoresis on a 2% agarose gel stained with ethidium bromide (1 µg/mL) for visualization under UV light. A DNA extraction kit from agarose gel (Fermentas) was used for purification of the PCR products. The purified PCR products were sequenced using an ABI 377 automated DNA sequence machine employing the same primers as used in the PCR. The sequences were aligned with the target one using the Basic Local Alignment Search Tool.

Analytical sensitivity and specificity of LAMP

A tenfold serial dilution prepared from extracted DNA from standard *C. albicans* PTCC 5027 strain from 1 ng to 1 fg was used to the analytical sensitivity of the LAMP technique. To determine the minimum amount of template DNA in any reaction that could be detected by each protocol, the dilution series were tested in duplicate with the LAMP technique. The LAMP specificity for *C. albicans* detection was also tested using the DNA samples of other clinical *Candida* species including *C. parapsilosis*, *C. krusei*, *C. collicolasa*, *C. holmic*, *C. tropicalis*, *C. kefyr*, *C. intermedia*, *C. mondi*, *C. glabrata*, *C. famata*, and three non-*Candida* yeast, *Cryptococcus curvatus*, *Trichosporon asahii* and *Cryptococcus mucoides*.

Statistical analysis

The SPSS 27.0 statistical software for Windows (SPSS Inc., Chicago, IL, USA) was used for statistical analyses

of the obtained data. To compare the differences, the Chi-square test was used. All statistical tests were two sided and a p value less than 0.05 was considered to be statistically significant.

Results

Study population and clinical samples

In this study, clinical samples (70 yeast specimens) were collected previously over the period between September and December 2014 from hospitalized patients at the general hospitals of Lorestan province, West Iran. The study population included 19 (27.1%) males and 51 (72.9%) females. These 70 yeast samples were harvested from 4 vaginal samples, 60 urine culture samples, 1 blood sample, 1 sputum sample, 1 synovial fluid sample, 2 bronchoalveolar (BAL) fluid samples, and 1 abscess sample. After extraction, the DNA from all yeasts cultured using the DNA extraction kit (DNG-PLUS, Sinnagene, Iran) according to the manufacture's protocol, the quality (A260/230, A260/280 ratios) and quantity (ng/uL) of DNA were evaluated using electrophoresis and Nano spectrophotometer (WPA-Biowave II, England) that an average of 1.7 for A260/230, 1.9 for A260/280 ratios and 180 ng/uL for quantity of DNAs was obtained.

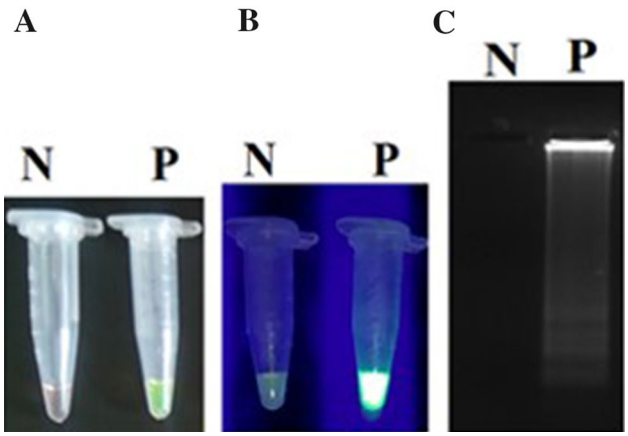

Fig. 1 Visualization of LAMP products with SYBR Green I **a** under natural light, **b** under UV light and **c** agarose gel electrophoresis. *N* no template control, *P* positive control

LAMP reaction

The positive LAMP reaction was visually recognizable by adding SYBR Green I to the reaction tubes, whilst the negative remained orange and the positive reaction turned green (Fig. 1a, b). The positive LAMP reaction also produced a characteristic ladder-like pattern of multiple bands of different sizes up to the loading well on a 1.5% agarose gel stained with ethidium bromide (Fig. 1c). Out of 70 yeast samples analyzed by the LAMP technique, 24 samples (34.3%) were positive and 46 samples (65.7%) were negative for *C. albicans.* In other words, 65.7% of the yeast samples analyzed by LAMP technique belonged to the other yeasts or other species of *Candida* (data not shown). Optimum results were obtained when the LAMP reaction time and temperature were maintained at 62 °C for 60 min for ITS2 genomic target. However, the result of the LAMP technique was also detectable at 62 °C from 45 min of reaction time (Fig. 2).

Comparison of the LAMP technique with conventional diagnostic methods for detection of *C. albicans*

Comparison of the results of the LAMP technique with the results of conventional diagnosis methods for *C. albicans* revealed that the germ tube production, CHROMagar *Candida*, and the LAMP technique are 100% consistent with each other, while, the overall Kappa consistency between carbohydrate assimilation assay, chlamydoconidia

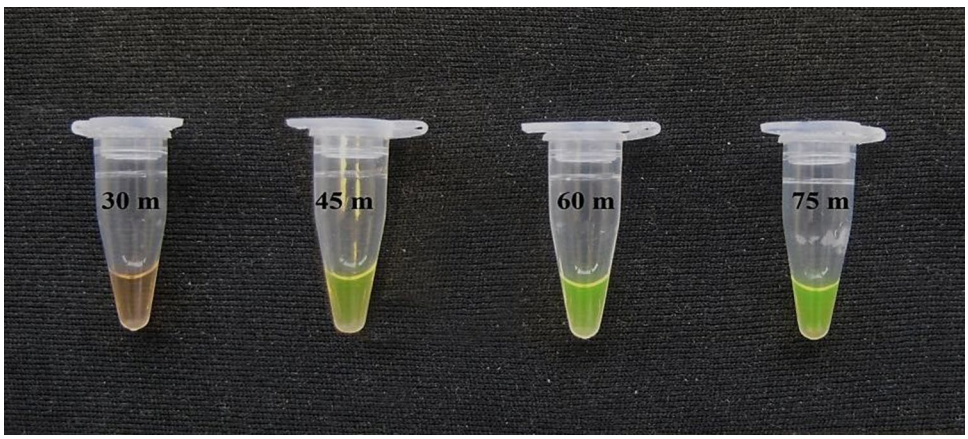

Fig. 2 Setup of the LAMP technique based on ITS2 gene target at 62 °C and different times

generation, and the LAMP technique was 0.968 and 0.392, respectively (Table 2).

Confirmation of the LAMP primer specificity

PCR amplification and subsequent sequencing of the products sequences using two outer primers of B3 and F3 (Table 1) were used to confirm the specificity of the LAMP primers for *C. albicans*. The results showed that the obtained partial sequences are identical to the corresponding ITS2 sequences of *C. albicans* reported in GenBank (accession numbers XR_002086439).

Analytical sensitivity and specificity of LAMP

A tenfold serial dilution of extracted DNA from standard *C. albicans* PTCC 5027 strain from 1 ng to 1 fg was used to evaluate the analytical sensitivity of the LAMP technique. The detection limits of the LAMP assay were 10 fg (Fig. 3), of the DNA from standard *C. albicans* PTCC 5027 strain. The DNA samples of other species of *Candida* and three non-*Candida* yeast were also used to evaluate the specificity of LAMP primers for *C. albicans*. No amplification was observed in the DNA samples of other yeast species and only the DNA sample of standard *C. albicans* PTCC 5027 strain was amplified (Fig. 4).

Discussion

C. albicans is one of the organisms most commonly isolated from clinical samples. In intensive care patients, *C. albicans* was responsible for 79% of candidemias (Chadwick et al. 2013; Lion 2017). Identification of *Candida* species using conventional detection methods has some limitations. Moreover, identifying some unusual species using these methods is difficult and sometimes impossible (Kim et al. 2002; Pincus et al. 2007). Rapid and accurate identification

Table 2 Comparison of the results of LAMP technique with common conventional assays for the detection of *C. albicans*

Diagnostic assay	LAMP technique						Kappa CO	*P* value
	Positive		Negative		Total			
	No	*%*	*No*	*%*	*No*	*%*		
Carbohydrate assimilation assay (ID 32C)								
Positive	23	95.9	0	0.0	23	32.85	0.968	< 0.001
Negative	1	4.1	46	100	47	67.15		
Total	24	34.28	46	65.72	70	100		
Germ tube production								
Positive	24	100	0	0.0	24	34.28	1	< 0.001
Negative	0	0.0	46	100	46	65.72		
Total	24	34.28	46	65.72	70	100		
CHROMagar Candida								
Positive	24	100	0	0.0	24	34.28	1	< 0.001
Negative	0	0.0	46	100	46	65.72		
Total	24	34.28	46	65.72	70	100		
Chlamydoconidia generation								
Positive	15	62.5	10	21.73	25	35.71	0.392	0.001
Negative	9	37.5	36	78.27	45	64.29		
Total	24	34.28	46	65.72	70	100		

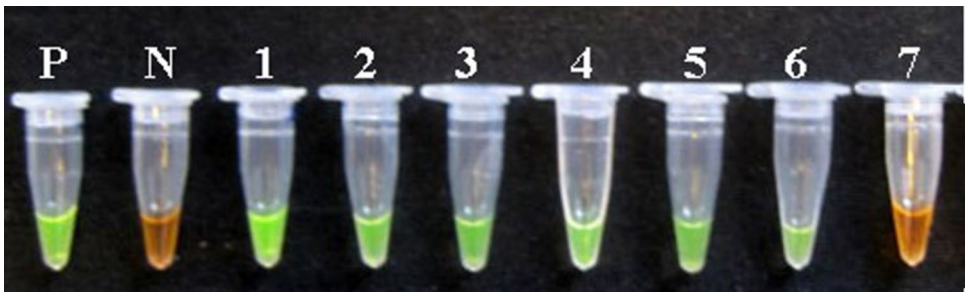

Fig. 3 Analytical sensitivity of the LAMP assay for the specific detection of *C. albicans* standard strain DNA based on the ITS2 gene amplification. P, positive control; N, negative control; lanes 1–7 represent 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg and 1 fg of DNA, respectively

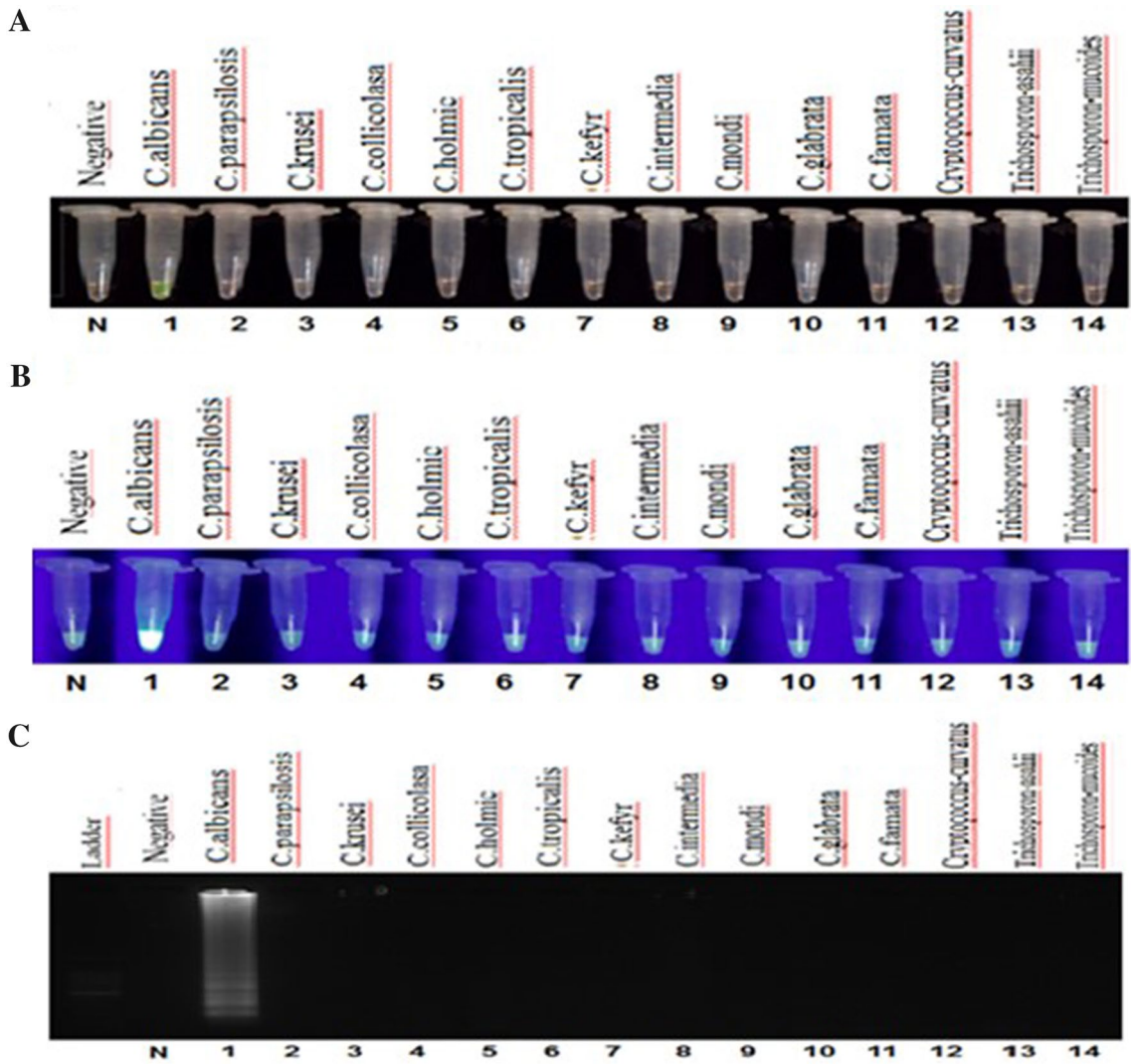

Fig. 4 Evaluation of the LAMP specificity for *C. albicans* detection using the DNA samples of other *Candida* species and three non-*Candida* yeasts. Ladder, 100 bp molecular-weight marker; N, no template control; 1, positive control; No. 2–14 represent other *Candida* species and three non-*Candida* yeasts

of *C. albicans* from a non-*albicans* species is essential for prevention and timely treatment of the disease, especially in patients with recurrent infection (Anaissie et al. 2009). In this study, out of 70 yeast samples collected from hospitalized patients, 24 samples were positive for *C. albicans* using the LAMP technique. These positive samples were the same ones that were positive for *C. albicans* by CHROMagar *Candida* and germ tube production methods, indicating 100% consistency (Table 2). In other words, the two conventional methods for *C. albicans* detection, CHROMagar *Candida* and germ tube production, are as effective as the LAMP technique in identifying the *C. albicans*. But, these methods cannot differentiate all *Candida* species and require a specialist to perform the test and the amount of inoculated organism, type of protein liquid, and temperature which are effective in the results of the test. Besides, the germ tube production method is not accurate and has false-negative results (Kim et al. 2002; Pincus et al. 2007; Anaissie et al. 2009).

In the present study, 62 °C and 60 min were the optimum time and temperature to the successful amplification of standard *C. albicans* strain DNA using the LAMP assay. However, the result of the LAMP technique was also detectable at 62 °C and 30 min of reaction time. This finding indicates that the LAMP technique is a rapid molecular assay for the detection of *C. albicans* in clinical samples. The analytical sensitivity of the LAMP assays against serial dilutions of the purified standard *C. albicans* strain DNA showed that the detection limit of LAMP is 10 fg of *C. albicans* DNA (Fig. 3). The high sensitivity of LAMP is due to the 2–3

couple of specific primers used in the reaction and the autocycling amplification resulting from *Bst DNA polymerase* can produce very large amounts of DNA of various sizes (Nagamine et al., 2002).

Of the 24 samples which were detected positive by LAMP technique, one sample was identified negative by carbohydrate assimilation assay (ID 32C), whereas all the 46 samples identified by LAMP technique negatively were also detected negative by carbohydrate assimilation assay, indicating a 98.5% agreement between the two tests for identifying *C. albicans* (Table 2). The minimum consistency rate (72.8%) was observed between the result of chlamydoconidia generation test and the results of LAMP assay for the detection of *C. albicans* (15 of 24 positive samples and 36 of the 46 negative samples which were detected by LAMP technique). Out of 247 yeast species studied by Mirhendi and colleagues, 185 samples (66.5%) were detected positive for *C. albicans* using CHROMagar *Candida* method. Also, the comparison of results of the CHROMagar *Candida* method with the results of PCR method indicated that the results of the two methods were in perfect agreement with each other.[1] In another study conducted by Taghipour et al. (2011), the CHROMagar *Candida* method was able to detect 62 samples (69.7%) of 89 yeast species isolated from the patients as *C. albicans* (Taghipour et al. 2011).

Geographic conditions and different climate zones of Iran, on the one hand, and different cultures, socioeconomic conditions and different food habits in Iran from the other side resulting in various studies using a variety of traditional and new methods on different infectious diseases occur in this area (Badparva et al. 2009; Zebardast et al. 2014; Fallahi et al. 2016; Mahmoudvand et al. 2016; Shirkhani et al. 2016; Rashno et al. 2016; Rashno et al. 2017; Fallahi et al. 2017; Rostami et al. 2018; Fallahi et al. 2018). The current detection methods of *C. albicans* (direct microscopic examination and culture) have some limitations including the need for a long time to detect a fungal infection that results in false-negative results, the high costs of diagnosis and treatment (Shirkhani et al. 2016), indicating the need for suitable complementary methods in terms of speed, sensitivity, and cost-effectiveness. In the current study, comparison of the result obtained by LAMP technique and the results of other conventional diagnostic methods for *C. albicans* showed that the LAMP technique could detect *C. albicans* with relatively equal sensitivity and specificity to routine diagnostic tests for the detection of *C. albicans* without the disadvantages and shortcomings of these assays. The LAMP method can detect infection with high sensitivity, and specificity without the need for expensive equipment in the shortest possible time. In 2008, Inacio et al. used the LAMP method to identify the different species of *Candida* and reported that the LAMP technique is able to identify the different *Candida* species and *Saccharomyces* with high sensitivity and efficacy (Inácio et al. 2008). Kasahara et al. (2014) used a multiplex LAMP assays to detect medically important yeasts in dairy products. Based on their results, the LAMP assays could detect the medically important yeasts at a range between 10(0) and 10(3) cells mL (-1) in a contaminated dairy product within 1 h. They introduced the multiplex LAMP assays as a primary screening method for yeast contamination in food products. Sun et al. (2010) evaluated the LAMP assay for the rapid diagnosis of *Penicillium marneffei* (*P. marneffei*) in archived tissue samples. They reported that the LAMP test led to the specific identification of *P. marneffei* using pure cultures without cross reactivity to other fungi including other biverticillate penicillia. The detectable DNA limit for LAMP assay was reported as two copies.

Conclusion

Based on the results, it can be concluded that the LAMP method is as specific and precise as common diagnostic methods, but is faster, easier deployable or more sensitive. Therefore, this method can be used as a suitable complementary assay for *Candida* diagnosis in medical diagnostic laboratories and field conditions.

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Compliance with ethical standards

Conflict of interest The author declares no conflict of interest.

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