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Full Length Research Paper

LOOP mediated isothermal *AMP*lification (LAMP) in diagnosis of neorocryptococcosis

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As a result of the importance of quick diagnosis of *Neurocryptococcosis* in patients, generally, molecular techniques are preferred rather than traditional methods for their rapidity. In this research, we made an effort to design and optimize *L*oop mediated isothermal *AMP*lification (LAMP) technique using specific primers for *ura5* as the target gene for detection of *Cryptococcus neoformans* and *Cryptococcus gattii* beside comparison of this assay with the common molecular technique of polymerase chain reaction (PCR). We collected a quantity of 35 serum samples of HIV-positive patients and a number of 107 cerebrospinal fluid (CSF) samples of patients who had shown symptoms of meningitis. We designed target specific primers for PCR and LAMP techniques to trace *C. neoformans* and *C. gattii*. From the total 142 clinical specimens, five had positive results from LAMP technique, that is, two out of 107 CSF samples and three out of 35 serum samples were positive for *Cryptococcosis*, but all specimens had negative results by PCR technique. According to the results of this research, it is clear that LAMP technique detects the agent faster and its specificity and sensitivity are much higher. Results reveal that LAMP as compared to PCR can find the target even in those samples containing a fewer number of the agents. LAMP is even more beneficial than PCR and it can be applied just using a simple thermal block or water bath. Therefore, we recommend diagnosis of *Neurocryptococcosis* by using LAMP technique which is useful in clinical and ambulatory laboratories.

Key words: Cryptococcus, *L*oop mediated isothermal *AMP*lification (LAMP), neurocryptococcosis, polymerase chain reaction (PCR).

INTRODUCTION

From molecular, biochemical, ecological and epidemiological aspects, the pathogenic *Cryptococcus* species complex is divided into two distinct species, *Cryptococcus neoformans* and *Cryptococcus gattii* (Casadevall A and Perfect JR 1998). *C. neoformans* is a capsulated yeast and proliferates by budding and sexual reproduction (Mitchell, 2003). *C. neoformans* has three serotypes, A/B/AD according to its polysaccharide capsular antigens (serotype AD is a hybrid which has both antigens existing on serotype A and B) (Boekhout et al., 2001). Serotype B and C belong to other similar but distinct species, that is, *C. gattii* (Kwon-Chung and Varma, 2006; Enache-Angoulvant et al., 2007). Members of *Cryptococcus* species complex are opportunistic pathogens responsible for frequent fatal cases of meningoencephalitis (Lucas et al., 2010). Occurrence of phenotypic variations of serotype A and D in chronic infections can result in resistance to phagocytosis (Guerrero et al., 2006). Recently, serotypes A and D are introduced as *C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans*, respectively (Nielsena et al., 2007). Phylogenic analysis reveals at least six cryptic species defined as molecular types (VNI/II/B, VNIV, VGI,

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VGII, VGIII and VGIV). The pathogenic *Cryptococcus* species complex is clustered in Filobasidiella clade within the order Tremellales (Findley et al., 2009). *C. neoformans* is an opportunistic infection mostly in immunocompromised patients. In recent decades, *Cryptococcosis* has shown specific importance in public health level (Bialek et al., 2002; Pappalardo and Melhem, 2003; Barreto de Oliveira et al., 2004). *C. neoformans* after entry through inhalation and localization in lungs proliferates and shows tendency to infect central nervous system (De Souza et al., 2004). Use of more specific and sensitive techniques for diagnosis of *Neurocryptococcosis* is necessary for rapid fungal therapy and prevention of patient's death (Paschoal et al., 2004; McTaggart et al., 2011). Due to disadvantages of known traditional methods which are time consuming and inefficient, recent molecular techniques such as polymerase chain reaction (PCR) are preferred. According to the extensive investigation on the PCR technique and its more precise results on diagnosing different pathogens, it is clear that it has more acceptable advantages, as compared to traditional diagnostic methods like Nigrosin staining and culture based methods, but its need of expensive instruments and complexity of procedure causes seeking for techniques that are more economical and simpler. *L*oopmediated isothermal *AMP*lification technique can be a suitable substitute for PCR. LAMP technique is a simple and very rapid method published by Notomi et al. (2000). In LAMP technique, target DNA replication occurs in isothermal conditions, so LAMP technique is very fast because, in contrast to PCR, there is no time delay for changing temperature levels (Notomi et al., 2000; Mori and Notomi, 2009). In this technique, steps of DNA denaturation and DNA replication occur simultaneously. There are four primers in this technique [two internal primers (FIP and BIP) and two external primers (F3 and B3)] which recognize six regions of template DNA. The LAMP technique was improved when Nagamine et al. (2002) added two more primers called *Loop primers* to the reaction, so DNA amplification went faster. These two specific loop primers plus previous four primers precisely recognized eight regions of target DNA. So, this technique should have much higher specificity and sensitivity as compared to other known DNA amplification systems (Mori et al., 2001; Nagamine et al., 2002). Products of LAMP reaction are DNA fragments with stemloop structures and DNA fragments with several palindromic sequences and also cauliflower structures of DNA with several loops (Notomiet al., 2000; Parida et al., 2008). In this situation, a huge amount of amplified DNA (10 to 30 µg in 25 µl) can be produced conserving high specificity in a short time (15 to 60 min) (Mori et al., 2001; Mori et al., 2006). In LAMP technique, it is possible to trace amplified DNA in shorter time by adding SYBR Green I and inspecting it under UV light (Moslemi, 2009). In this study, LAMP technique was developed for detection of *C. neoformans* and *C. gattii* and results were compared with those of PCR technique.

MATERIALS AND METHODS

Positive and negative controls

A standard strain of *C. neoformans* (ATCC number: 13690) and a strain of *C. neoformans* that had been isolated from a patient with Cryptococcosis using microscopy (Indian Ink method) confirmed by culture on Niger seed agar (AcumediaCompany), at Resalat diagnostic laboratory of Tehran were used as positive control. Both were maintained by cryopreservation at -20°C. Then, DNA was extracted from isolated brown colonies of *C. neoformans* by applying DNP-kit (CinnaGen Co. Iran) in order to be used as positive control in amplification reaction. Water was used as negative control.

Specimen collection

142 suspected clinical specimens were collected including 107 suspected cerebrospinal fluid (CSF) samples from Shahid-Bahonar hospital of Karaj and 35 HIV-positive serum samples from Virology Department of the Pasteur Institute of Iran. These latter samples had been identified and confirmed by serological methods, and had been collected in 2006 to 2007 from untagged remnants of samples from clinical laboratories in Tehran, Iran. All specimens were carried to the laboratory under frozen condition.

DNA extraction

All samples underwent DNA extraction using DNP-kit (CinnaGen Co. Iran Art: DN8115C). The procedure was as follows: At first 100 εl of the sample was mixed in a microtube with 5 εl of "protease" and incubated at 72°C for 10 (min), then 400 εl of "DNA lysing buffer" was added and tube was spun for 15 to 20 s, in this step, all aggregations were mixed well. Then 300 εl of "precipitating solution" (isopropanol) was added and the tube inverted 10 times. Then, the tube was put into the -20°C freezer for 20 min, followed by centrifugation in 12000 rpm for 10 min. Then, the supernatant was decanted and 1 ml of "washing buffer" (cold 70% ethanol) was added and the tube was centrifuged at 12000 rpm for 5 min. Then, the supernatants were decanted. The tube was heated at 65°C for 5 min until its sediment dried out. Finally, to wash the wall of the tube, 30 εl of sterile double distilled water was added, then the tube was warmed in 65°C incubator again for 5 min to prepare the extracted DNA suspension.

Primer design

C. neoformans specific LAMP primers for *ura5* gene on chromosome 7 were designed. Specific PCR primers were also designed for chromosomal locus of rDNA gene on chromosome 2 (Mitchell et al., 1994; Paschoal et al. , 2004) (Table 1). Above mentioned target sequences are located on *C. neoformans* and *C. gattii* genome, so both species can make a positive result in both techniques.

PCR reaction

PCR reaction was optimized by applying extracted DNA of the standard strain (ATCC number: 13690) of *C. neoformans*. The total

Table 1. Primer sequences designed for PCR and LAMP techniques.

Primer	Sequence
PCR	
ITS1	5'TTCGGCACGTTTTACACAAAC3'
CN4	5'ATCACCTTCCCACTAACACATT3'
LAMP	
F3	5' TCCTTGGCTGCTGTCTCC 3'
B3	5' GCCTTGCCAGAGGTAAGAAC 3'
FIP	5'TGGGACAGACTCACGTCCTTCTAAACCGGCAAAGATATCGGC 3'
BIP	5' CCAGTGCGACAGCGATGAGCTTCGTCCCTTGAGAGGCG 3'
LF	5' TCTCCTTCCTGTTGTAGCAGTA 3'
LB	5' AGCCAGTAGCACGGTGAGGG 3'

ITS1 and CN4 are used as forward and reverse primers in PCR. LAMP primers are designed by primer explorer V4 software (Primer explorer V4; http://primerexplorer.jp/e/). PCR, polymerase chain reaction; LAMP, *L*oop mediated isothermal *AMP*lification.

volume of the reaction was 25 εl. Substrates of the reaction were as follows: 5 εl of extracted DNA, forward and reverse primers with final concentration of 0.4 εM, 1X PCR buffer (CinnaGen Co. Iran), magnesium chloride ($MgCl_2$) with final concentration of 1.5 mM (CinnaGen Co. Iran), dNTPs with final concentration of 0.2 mM (CinnaGen Co. Iran) and two units of Taq DNA polymerase (CinnaGen Co. Iran). The thermal cycler was programmed for one initial cycle for denaturation step at 94°C for 6 min, then 40 cycles each including one denaturation step at 94°C for 30 s, annealing step at 62°C for 30 s and polymerization step at 72°C for 1 min. In addition, at the end of the program, a final extension step was adjusted at 72°C for 10 min.

LAMP reaction

LAMP reaction was optimized by application of extracted DNA of the standard strain (ATCC number: 13690) of *C. neoformans*. The LAMP reaction was set up for a total volume of 25 εl. 5 εl of extracted DNA was amplified in the presence of the following reagents: Mixed primers including internal primers (FIP and BIP) each one with final concentration of 1.6 εM and external primers (F3 and B3) each one with final concentration of 0.2 εM, Loop primers (LF and LB) each one with final concentration of 0.8 εM, $Mgso_4$ with final concentration of 7.2 mM, Betain (Sigma-Aldrich) with final concentration of 0.8 mole (M), dNTPs with final concentration of 1.4 mM (CinnaGen Co. Iran) and enzyme buffer with final concentration of 1X, plus eight units of Bst polymerase (New England Biolabs). The reaction was run in one single cycle at 66°C for 60 min. Positive and negative controls were applied for each round of examination.

Evaluation of PCR and LAMP reactions

PCR products together with positive and negative controls were run in 1.5% agarose gel-electrophoresis containing ethidium bromide and 0.5X buffer, then inspected using UV light (300 nm) transilluminator (UVP), the diagnostic criteria for PCR method observing 415 bp product on gel-electrophoresis. LAMP products were stained with 1 εl of 0.1% SYBR Green I (Invitrogen cat no: S-7563) and was evaluated using UV light (300 nm) transilluminator (UVP), the diagnostic criteria was observing florescent green color in tube under UV light.

PCR and LAMP sensitivity tests

In order to determine the sensitivity of these techniques in this research, the standard strain of *C. neoformans* (ATCC number: 13690) was applied in preparing a series of dilutions from 10 million copies to five copies of DNA to undergo both PCR and LAMP reactions.

PCR and LAMP specificity tests

To determine the specificity of both techniques, extracted DNA of mouse, human, *Escherichia coli*, *Saccharomyces cerevisiae*, *Toxoplasma gondii*, *Candida albicans*, *C. neoformans* and cDNA of Hepatitis C virus were applied in PCR and LAMP reactions using the above-mentioned primers.

RESULTS

LAMP positive products

In LAMP technique, color of positive results were observed to be florescent green and negative results have shown unchanged pale color of SYBR Green I (Figure 1A).

PCR positive products

According to gel-electrophoresis of PCR products, positive control product was 415 bp (Figure 1B).

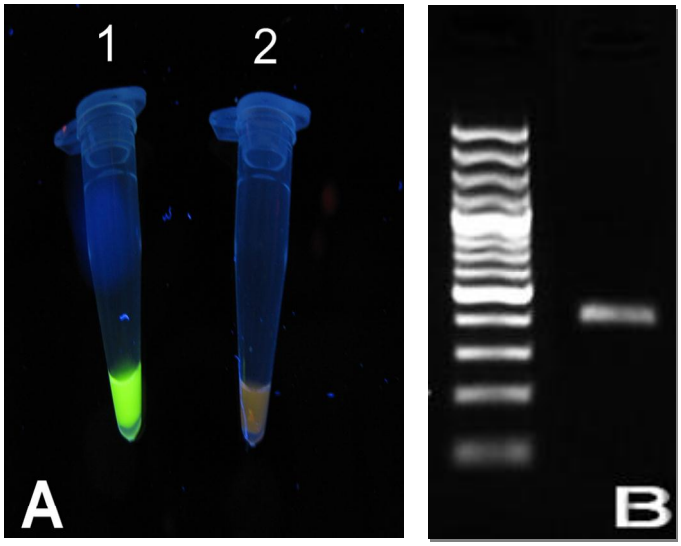

Figure 1. PCR and LAMP products. A) LAMP: 1, positive reaction; 2, negative reaction. B) Electrophoresis: 1, 100 bp DNA ladder; 2, PCR product (415 bp). PCR, Polymerase chain reaction; LAMP, *L*oop mediated isothermal *AMP*lification.

Sensitivity and specificity

Sensitivity and specificity tests had acceptable results for both techniques. Sensitivity tests for PCR technique had positive results in the presence of few numbers of 50 copies of targeted DNA. Meanwhile, sensitivity tests for LAMP technique had positive results for as little as five copies of targeted DNA presented in the reactions (Figure 2).

In specificity tests, both techniques resulted in positive reactions with *C. neoformans* and negative reactions with other organisms (Figure 3).

Clinical samples

In PCR

From 142 samples, all of them had negative results in PCR technique.

In LAMP

From 142 samples, five (3.5%) have shown positive results in LAMP technique, that is, two samples (1.85%) were positive from 107 CSF specimens of suspected patients and three samples (8.6%) were positive from the HIV-positive serum specimens.

Statistical analysis

Results of these two techniques were analyzed using Minitab-16 software. According to two proportions test, differences were significant (P-value = 0.012), because of the small number of samples, Fisher's exact test was applied also and the differences were significant (P-value = 0.030). Two proportion test was applied again for analysis of the LAMP results of two groups of specimens (serum and CSF) and differences were significant (P-value = 0.031).

DISCUSSION

Symptoms of cryptococcal meningitis are like the symptoms of other meningeal infections, so early diagnosis of cryptococcal meningitis is necessary for effective therapy (Satpute et al., 2006). Previous studies have shown that 10 to 20% of HIV positive patients are infected with *C. neoformans* (Steenbergen and Casadevall, 2000; Jackson and Hosseinipour, 2010). Although, generally it has been thought that *Cryptococcosis* is just associated with immunocompromised patients, but recently frequent reports show occurrence of *Cryptococcosis* in immunocompetent individuals (Hoang et al., 2004; Satpute et al., 2006). Hence, in this research, specimens of immunocompetent patients were used as well. The importance of these infectious agents is wide spread because the agents are widely distributed in the world and they are isolated from pigeon droppings and eucalyptus trees (for *Cryptococcus gattii*). In addition, the route of infection is through inhalation (Klein et al., 2009; Jackson and Hosseinipour, 2010). Different traditional diagnostic methods have already been used but most of them like culture on Niger seed agar (Pedroso et al., 2007; Mseddi et al., 2011) and canavanineglycine-bromothymol blue medium (Klein et al., 2009) or serologic tests like latex agglutination test) do not show high sensitivity, specificity and rapidity. Up to now, some molecular techniques are designed to diagnose these agents and most of them are PCR based methods (Martin et al., 2000; Velegraki et al., 2001; Playford et al., 2006; Enache-Angoulvant et al., 2007; Ito-Kuwa et al., 2007; Feng et al., 2008; Kaocharoen et al., 2008; Lusia et al., 2008). Although, these molecular techniques are more precise than abovementioned traditional methods, there are some disadvantages like need for applying expensive instruments and need for complex and time consuming amplicon detection methods. In this study, PCR electrophoresis technique as a gold standard for molecular diagnosis of these agents is compared with the novel LAMP technique. Some advantages of LAMP technique are as follows: 1) LAMP reaction is less sensitive to suppressive substances present in biological specimens (Mori and Notomi, 2009). 2) Amplicon detection in LAMP technique is much faster and simpler (Parida et al. , 2008). 3) The LAMP technique is capable of amplifing target DNA in isothermal condition with high

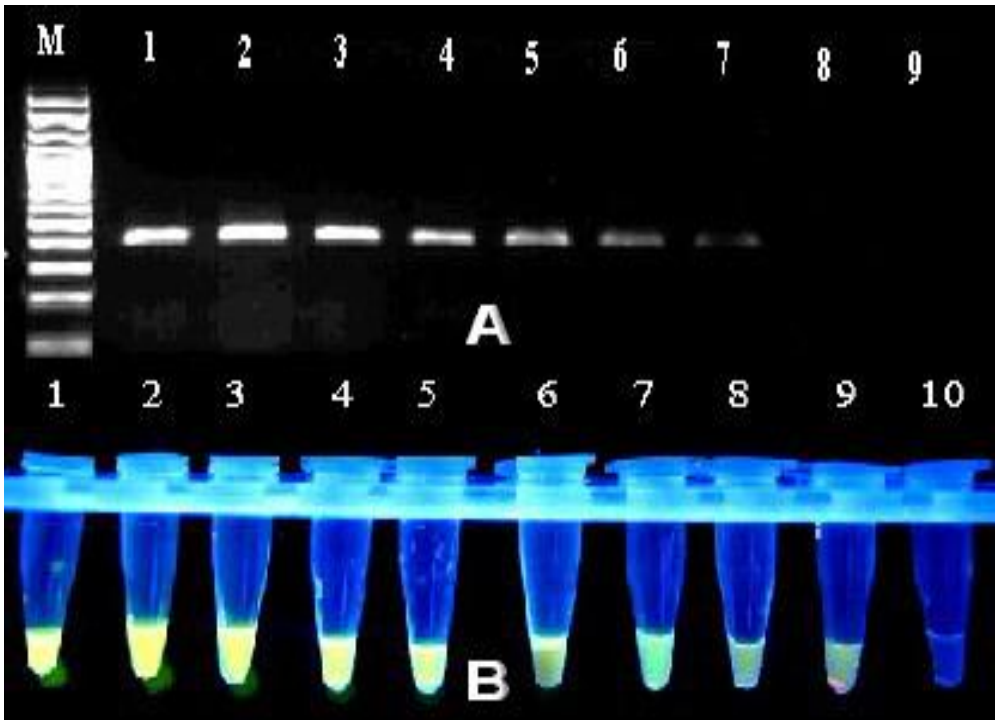

Figure 2. PCR and LAMP sensitivity tests. A, PCR; M, 100 bp DNA ladder, copy number of DNA: 1 = 10^7, 2 = 10^6, 3 = 10^5, 4 = 10^4, 5 = 10^3, 6 = 10^2, 7 = 50, 8 = 5 and 9 = control$^{(\delta)}$. B, LAMP; 1 = control $^{(+)}$, copy number of DNA; 2 = 10^7, 3 = 10^6, 4 = 10^5, 5 = 10^4, 6 = 10^3, 7 = 10^2, 8 = 50, 9 = 5 and 10 = control $^{(\delta)}$. PCR, polymerase chain reaction; LAMP, *L*oop mediated isothermal *AMP*lification.

efficiency and without influence of nontarget DNA presented in the reaction, for this reason LAMP reaction is applicable even with a simple thermal block or water bath and do not require expensive equipments like thermal cycler, etc. (Notomi et al., 2000). 4) The LAMP reaction is so specific for the target sequence that it can recognize and amplify eight distinct regions of the target sequence even in small amounts (e.g. five copies of target DNA) using six specific primers (F3, B3, FIP, BIP, LF and LB) (Nagamine et al., 2002). In this study, the specificity of the LAMP primers were checked using Basic Local Alignment Search Tool in NCBI website (http://blast.ncbi.nlm.nih.gov), the best query coverage and E-value for both *Cryptococcus* species so the hypothesis of false-positive results is eliminated. From the obtained results of this study, it is apparent that the occurrence of the Cryptococcal meningitis in HIV positive patients (8.6%) are more than its rate in non HIV infected individuals (1.85%). Deficiency in cellular immunity related to CD^{4+} and inability to omit the invading agent increase the probability of infection in HIV positive patients. There are two reasons why the positive results of LAMP technique were false negative in PCR technique: 1) lower efficiency and specificity of PCR primers in detection of two sequences in the target, as compared to six sequences of targeted DNA by LAMP primers. 2) The existence of such a few numbers (below 50 copies) of DNA in specimens that were undetectable by PCR technique (according to results of sensitivity tests). Therefore, rapid and precise LAMP detection of *C. neoformans* starts the application of emergent and effective therapy and improvement in clinical care strategy in order to prevent the risk of death in patients. It can also speed improvement of epidemiological investigations.

Up to now, many successful studies are been published about LAMP detection of different bacterial, viral, fungal and parasitic agents. Until 2009, more than 180 reports evaluating LAMP technique were documented (Mori and Notomi, 2009). Growing improvement of this technique from year 2000 until now has introduced it as a genetic point of care system (gpoct). Sensitivity and specificity of this technique are improved by using loop primers (LF and LB) (Nagamine et al., 2002). Just by applying a cost-effective photometer can measure insoluble magnesium pyrophosphate realtime turbidity produced in the course of the amplification process (Mori et al., 2004). Recently, a novel integrated isothermal device for realtime turbidity measurement of LAMP reaction has been introduced (Lee et al., 2008).

Today, some efforts are been made to use the LAMP technique in Labonachip technology (Whitesides, 2006; Nakamura et al., 2007).

LAMP technique as compared to PCR based

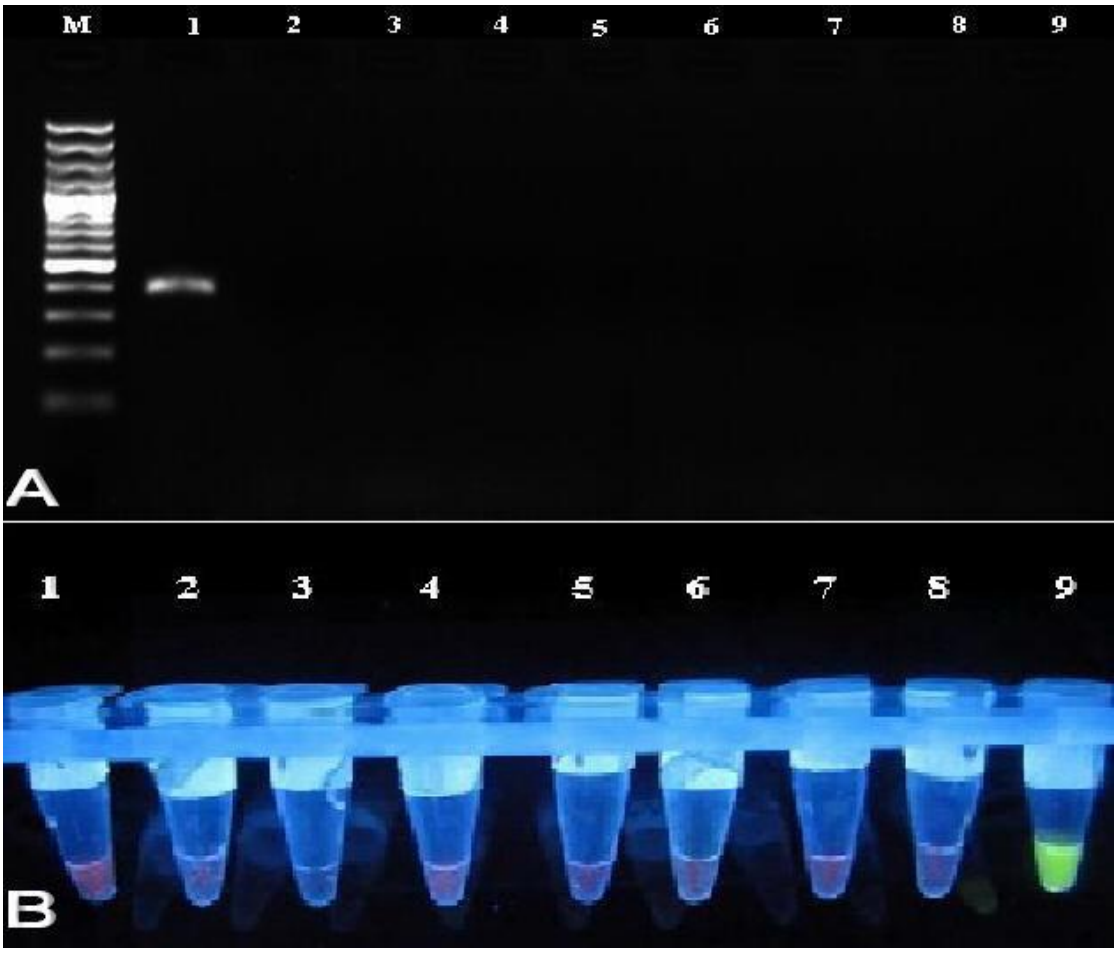

Figure 3. Specificity test for PCR and LAMP. A, PCR; M, 100 bp DNA ladder, DNA of: 1) *C. neoformans*; 2) mouse; 3) human; 4) *E. coli*; 5) *S. cerevisiae*; 6) *T. gondii*; 7) *C. albicans*; 8) HCV; 9) control(ð). B, LAMP; Tube 1) control (ð); 2) mouse; 3) human; 4) *E. coli*; 5) *S. cerevisiae*; 6) *T. gondii*; 7) *C. albicans*; 8) HCV; 9) *C. neoformans*.

techniques has shown the ability of detecting fewer particles of *C. neoformans* or *C. gattii* in specimens more efficiently and rapidly by applying six specific primers, especially in diagnosis of cryptococcal meningitis. On the other hand, application of this laboratory technique as compared to PCR techniques is much simpler and cost - effective that it can be performed just using a simple thermal block or water bath, and there is no need for expensive equipments like thermal cycler. Therefore, this study suggests that LAMP as a novel technique can be a suitable substitute for PCR techniques to detect *Cryptococcosis* in diagnostic and ambulatory laboratories in developed or developing countries where few instruments are available.

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Abbreviations:

LAMP, *L*oop mediated isothermal *AMP*lification; **PCR,** polymerase chain reaction; **CSF,** cerebrospinal fluid.

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