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Assessment and Comparison of LAMP and PCR Techniques in Diagnosis of *Cryptococcus neoformans*

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The purpose of this study is comparative assessment of traditional methods and two molecular techniques in diagnosis of *C. neoformans* in cerebrospinal fluid (CSF) as well as culturing samples. 300 CSF samples from patients with suspected meningitis have been collected from multiple hospitals. Samples were evaluated by staining, culture and PCR and LAMP. The target gene of primers used in this study for PCR, selected on the basis of 16S rRNA, also, the LAMP test primers were designed with the help of "Primer explorer V4" software based on the ura5 gene. Internal Control of PCR constructed by the competitive strategies and cloned in pTZ57R. The PCR results were positive in 4 cases, while the results of culturing and staining tests were negative for all the CSF samples. The sensitivity of LAMP test was determined up to 5 cells of *C. neoformans*. The LAMP test was positive in 5 cases of CSF samples (four positive cases of PCR and another sample with a negative PCR). The LAMP technique which provides additional advantages such as higher sensitivity and specificity and no need to expensive hardware, also eliminates the identification stage by electrophoresis methods, and available especially in developing countries.

Key words: PCR, LAMP, *Cryptococcus neoformans*.

C. neoformans is a yeast member of Sporidiobolaceae family with a polysaccharide capsule which often replicates by budding[1]. Based on the capsular polysaccharide antigen, the fungi are categorized into five serotypes, including A, D, AD, B and C. This species composed of two varieties according to the molecular, biochemical, ecological and epidemiological differences namely: *C. neoformans* var. *neoformans* which includes A, D and AD serotypes and *C. neoformans* var. *gattii* which includes B and C serotypes[2,3]. Recently, scientists called the A serotype and the D serotype as *C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans, respectively*[4-5]. Studies suggest a close similarity between the *C. neoformans* var. *gattii* and the *C. neoformans* var. *neoformans* which composed of two non-pathogenic species namely *C. amylolentus* and *Tsuchiyaea wingfieldii* as well as *Filobasidiella depauperata*[6].

In the absence of strong immune system capability, Cryptococcosis acts as a human opportunistic infectant, furthermore in current decades, regarding to increasing of HIV positive people it has become very important in public health level[7]. Using more sensitive and specific laboratory techniques for rapid detection and early antifungal treatment in diagnosis of neurological cryptococcosis, is essential (8).

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C. neoformans infections spread out through inhalation of the dust contaminated with living fungal conidia *in the human*, and usually people with cellular immune system deficiency will be infected[9]. The common appearances of disease are as lung and cervical forms, but due to spreading out of fungi through the primary pulmonary centre, other various forms of disease were reported as cutaneous, mucosal, bone and visceral. The human pathogen species is *C. neoformans*, which prefers to attack the central nervous system, because of unknown reasons. The infection is spread out to these centers, by apparent or unapparent pulmonary infections[10].

Generally, the culture-based methods are time-consuming techniques with low sensitivity which require both adequate facilities and experienced specialists to analyze the results; in addition, there is no enough sensitivity in staining methods. Compared to aforementioned two methods, serological methods which are based on antigen - antibody reaction, as well as using monoclonal antibodies, and the flow cytometry methods have higher sensitivity and accuracy[11]. However, false positive and/or negative results could be achieved due to cross reactions. Thus, we need a method with high sensitivity; specificity and speed performance to identify this agent needed which has no any limitations of above methods.

Previous studies suggested; because of their higher sensitivity, specificity and precision, the diagnostic methods based on PCR and the loop mediated isothermal amplification (LAMP) test sound to be appropriate methods to detect pathogens such as *C. neoformans* today[8,12]. The level of PCR sensitivity and accuracy in some specific parts of *C. neoformans* genome recognition depends on several variables such as DNA extraction method, the target gene, the designed primers and the method of product identification which are different in various studies[13].

The prepared primers are generally designed for following common portions: 16S rRNA gene[14], capsular gene[15] or other targets such as phospholipase enzyme gene[16]. However, to design the species-specified primers, more emphasis was on the existing constant parts in the rRNA gene sequences of C. neoformans. Despite of positive features of PCR method, some disadvantages such as expensive equipments and other problems make its widespread usage difficult, especially in routine laboratories.

Because of various reasons PCR method could be replaced, especially in developing countries, with Loop-mediated isothermal replication technique (LAMP), as a suitable method in diagnostic purposes[17]. This technique (LAMP) is a simple and time saving method which totally identifies 6 or 8 regions in the DNA model. Typically, compared to the other existing DNA isothermal replication methods, this test has very high sensitivity and specificity[18, 19, 20], so that large amount of DNA (10 -30 micrograms in 25 µl) can be made in a short period of time (30-60 minutes) maintaining high specificity[21]. Nowadays, the LAMP method has been commercially used to identify various pathogens. It can be considered as a significant technique in developing countries[17].

Several diagnostic methods compared in many studies which have been conducted in the diagnosis of *C. neoformans*. For example, three cultural, serologic and PCR methods compared by Rappelli et al . According to the results, the sensitivity of cultural, serologic and PCR methods in diagnosis of C. neoformans are 67%, 89% and 98%, respectively[22]. Rezai *et al* 2012, to detect the fungi in the serum of HIV positive people, used LAMP method and reported four positive cases.

This study aims to assess two molecular techniques; PCR and LAMP as two very practical methods in diagnose of *C. neoformans* in the infected samples such as cerebrospinal fluid as well as culture samples and comparing them.

MATERIALS AND METHODS

C. neoformans culturing**:** The provided strain obtained from the Microbiological Collection Center of Scientific and Industrial Research Organization (ATCC number: 13690) and two provided strains from the Resalat laboratory, Tehran (isolated from patient) were cultured on the Niger seed agar medium (Acumedia Company) and the plates were incubated for a week at 37 ° C (*C. neoformans* colonies usually grow within three days). DNA was extracted from three strains and the PCR and LAMP tests were optimized on them.

Sample collection

300 samples of cerebrospinal fluid (at least 500 µl) from patient suspected to meningitis have been collected during 2012-2013, which then transferred to the laboratory under cold conditions. The samples were centrifuged at 8000 rpm for 2 minutes. Then, the supernatant fluid was slowly evacuated; and remaining sediment was solved in 120 µl PBS buffer and 40 µl of the prepared solution was placed on the lamel and mixed with a drop of Hindi ink. The sample was observed under microscope after placing a lamella on it, in case of existence of *C. neoformans* a transparent and clear zone can be seen around the yeast cells which appeared as a sky scene full of stars. Another 40 µl of the sediment were cultured on the Niger seed agar medium (Acumedia Company) as well, and the plates were incubated at 37 °C for a week (*C. neoformans* colonies usually grow within three days).

DNA extraction from cerebrospinal fluid samples

To extract the DNA, DNP Cinaclon kit (Cat: DN811540) was used according to the kit instructions. The genomic DNA of bacteria, yeast and humans used in this study for the characteristics tests was also extracted using same method.

Primer design for PCR and LAMP

The used primers in this study for PCR technique are 16S rRNA gene-specific primers of *C. neoformans* (Table 1) [23]. Also, the LAMP test primers were designed with the help of "Primer explorer V4" software based on the existing sequence in the ura5 gene (ACCESSION EU399581) (primer explorer V4; http://primer explorer JP. / E /) (Table 1).

PCR reaction

5 µl of the extracted DNA using the ITS1 and CN4 primers specific to16S rRNA gene with the final concentration of 0.4 µl (1 µl of 10 mM concentration) were duplicated in the final volume of 25 µl by mixing with the followings: 2.5 µl of PCR (10 X) (Cinaclon) buffer; 0.75 µl of magnesium chloride ($MgCl_2$) (of 50 mM concentration (Cinaclon)); 0.5 µl of dNTP mixture (10 mM) (Cinaclon) and 0.3 µl of Taq DNA Polymerase enzyme (Cinaclon). The thermal cycles consisted of a six-minute cycle at 94° C, 40 sequential cycles (including 30 seconds at 94 °C, 30 seconds at 62 °C and 1 minute at 72 ° C,) and a final polymerization cycle of 72 °C for 10 minutes. After ending the thermal cycles, the electrophoresis technique was used to observe the amplified DNA. The PCR product alongside the size marker and positive and negative controls was studied on 1.5% agarose gel by using ethidium bromide or SYBR Green I and UV light on the Transilluminator device.

Construction of internal control (IC) and Ideal density

To construct the competitive internal Control of PCR test, we re-designed and synthesized the forward and reverse primers of Cryptococcus PCR at 5' portion of the kinetoplast gene primers of *Leishmania* with the product size of 620 pairs (Table 1). Doing the PCR reaction to replicate the internal control fragment, 5 µl of *leishmania's* extracted DNA the front and rear IC primers with the final concentration of 0.4 mM were amplified in the final volume of 25 µl were used by mixing with the followings: 2.5 µl of 10X PCR buffer; 0.75 µl of magnesium chloride ($MgCl_2$ 50 mM); 0.5 µl of mixed dNTP (10 mM) and 0.3 µl of Taq DNA Polymerase enzyme (5 unit/). The thermal cycles consisted of 35 sequential cycles (including 30 seconds at 94 °C, 30 seconds at 60 °C and 1 minute at 72 °C,) and a final polymerization cycle of 72 ° C for 25 minutes. After ending the thermal cycles, the electrophoresis technique was used to observe the amplified DNA. By using Blue/White Screening the resulting construction was selected. The suitable clones finally extracted via plasmid extraction and PCR test.

Different concentrations of standard *C. neoformans* IC and DNA were examined In order to obtain the optimal density of the plasmid containing IC which not competing the target DNA, The DNA concentration of *C. neoformans* as well as the IC concentration used in the test was determined by a spectrophotometer at A260. For PCR test, 5 µl of the DNA obtained from the samples were mixed with 1 µl of IC, and was used in the PCR mixture.

LAMP reaction

The reaction was performed at the volume of 25µl, including the following reagents: a mixture of 5.2 µl twice-distilled water, 2.5 µl of enzyme buffer with the 10X concentration, 1 µl of a mixture of external primers (F3/B3) with a concentration of 0.2 µM and internal primers (BIP/ FIP) with a concentration of 1.6µM, 1 µl of loop

primers (LB/LP) with a concentration of 0.8 µM, 1.8 µl of MgSO4 with a concentration of 7 mM, 1 µl (8U) of Bst DNA polymerase enzyme (New England biolab), 4 µl of Betain (Sigma) with a concentration of 0.8 M, 3.5 µl of dNTP with a concentration of 1.4mM and 5µl of the extracted DNA. The reaction was performed at the temperature of 66° C within 60 minutes. Positive and negative controls were used in each round of the reaction. To evaluate the reaction product, 1 µl of 10 times diluted SYBR green (Invitrogen lot: 49753A) was added to each reaction tube and was reviewed on the Transilluminator device at the 302 nm wavelength. The tubes containing positive and negative reactions were observed having fluorescent green and orange colors, respectively.

Sensitivity and specificity of LAMP and PCR reaction

To determine the sensitivity of optimized LAMP and PCR tests, different dilutions of the *C. neoformans* in deionized water were prepared in the light of direct counting microscopic method using extracted DNA from different dilutions. Further, to determine the specificity of the optimized PCR and LAMP tests, their functions assessed on the DNAs of human, mice, *Candida albicans*, *Aspergillus niger*, *Escherichia coli*, *Fusarium solani*, *Saccharomyces cerevisiae*, *Pichia pastoris* and a number of other agents, along with positive and negative control samples.

PCR product and internal control Cloning

After PCR product and IC purification, both amplicons were cloned at pTZ57R vector using the T/A cloning Thermo scientific kit(K1214). The resulting plasmids were extracted by the Plasmid Mini Extraction Kit of Bioneer Company(K3112). Then, the plasmids containing the PCR product and the IC were confirmed, using PCR method.

Sequence finding

Using "Dideoxy Chain Termination" method by a Macrogen inc, DNA sequencing was conducted in two directions

RESULTS AND DISSCUSSION

The PCR test was optimized on DNA extracted from the *C. neoformans* strain (cultured on the medium) (Figure1A). The diagnosis limitation (sensitivity) was obtained at least as 10 copies in the examined sample by preparation series of dilutions of the yeast culture and performing DNA extraction and PCR test (Figure 1B). Also, no unwanted product was seen in the specificity test.

In Figure 2, the results of internal control PCR test and its examination by different DNA amounts of *C. neoformans* are seen which have variety of 100 to 3 million fungi. The IC ideal number for PCR test was obtained as 1000 per reaction and then used for disease samples examination.

To diagnosis of Cryptococcus infections in the cerebrospinal fluid samples, specific ITS1 and CN4 primers were used. While the results of culturing and staining tests were negative for all the CSF samples PCR test results were positive in 4 cases of 300 CSF samples. To review and approve the replicated PCR product in the disease sample, the 415-bp fragment was sequenced by "Sequencing" method. As well, nearly 100 percent compliance appeared by comparing the resulting sequence with existing sequences through BLAST application.

Table 1. Used primers in PCR and LAMP tests and making internal control

Primer	Sequence (5'…….3')
ITS1	5' TCC GTA GGT GAA CCT GCG G 3'
CN4	5' ATC ACC TTC CCA CTA ACA CAT T 3'
ICF	5' TCC GTA GGT GAA CCT GCG G -TCG CAG AAC GCC CCT ACC 3'
ICR	5' ATC ACC TTC CCA CTA ACA CAT T -AGG GGT TGG TGT AAA ATA GGC 3'
F3	5' TCC TTG GCT GCT GTC TCC 3'
B3	5' GCC TTG CCA GAG GTA AGA AC 3'
FIP	TGG GAC AGA CTC ACG TCC TTC TAA ACC GGC AAA GAT ATC GGC 3'5'
BIP	5' CCA GTG CGA CAG CGA TGA GCT TCG TCC CTT GAG AGG CG 3'
LF	5' TCTCCTTCCTGTTGTAGCAGTA 3'
LB	5' AGCCAGTAGCACGGTGAGGG 3'

The LAMP reaction was optimized on extracted DNA from the standard strain and two isolated strains from patients at a temperature of 66 ° C within 1 hour (Figure 3). The sensitivity of LAMP test with optimized parameters was determined up to 5 cells of *C. neoformans* (Figure 4). Also, very high specificity appeared in the LAMP test, since it acts specifically using six specific primers so that it only reacted with *C. neoformans* DNA and no reactions occurred with DNA of other used agents (Figure 5).

In 5 cases of CSF samples, the LAMP test was positive (four positive cases of PCR and another sample with a negative PCR).

Early and accurate diagnosis of infectious agents are Factors which reduce mortality, morbidity and the costs of hospital infectious diseases as well. Although the traditional

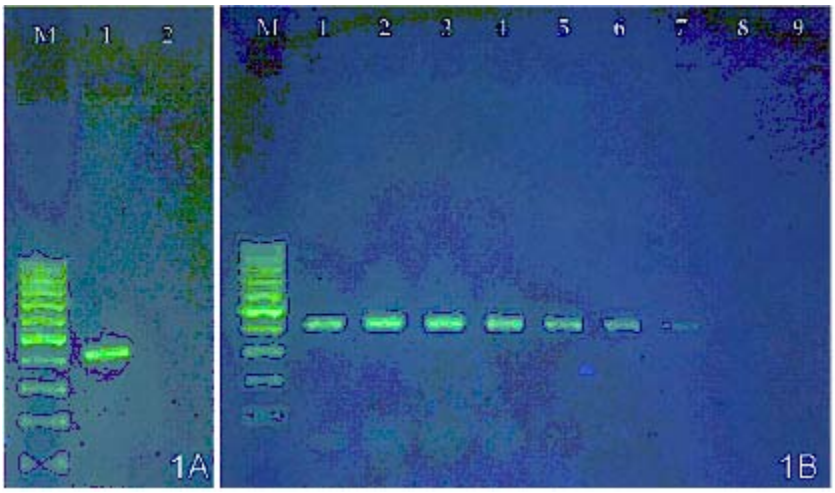

Fig. 1(a). The optimized PCR test on the standard *Cryptococcus neoformans* strain
M line: Fermentas size marker (100 bp DNA Ladder); Line 1: Positive PCR test;
Line 2: Negative control;
Fig. 1(b). PCR sensitivity test using counted colonies of *Cryptococcus neoformans* (indicated CFU)
M line: Fermentas size marker (100 bp DNA Ladder); Line 1: Positive control samples;
Lines 2-6: Contain certain samples, including, respectively: 2: (CFU 100000), 3: (CFU 10000), 4: (CFU 1000), 5: (CFU 100), 6: (CFU 10), 7: (CFU 5), 8: (CFU 1), 9: Negative control sample

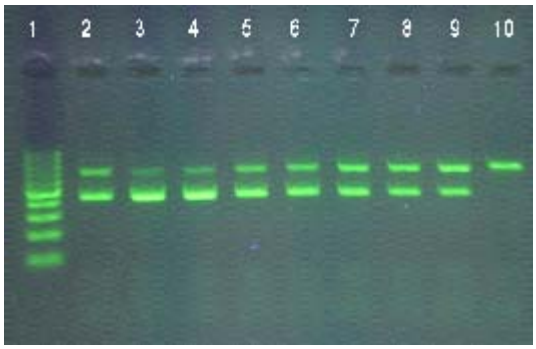

Fig. 2. The PCR electrophoresis results with certain number of *Cryptococcus neoformans* DNA:
1: Fermentas 100 bp size marker; 2: Positive control (containing IC and Cryptococcus DNA); 3: DNA of a number of 3000000 agents; 4: A number of 2000000; 5: A number of 1000000; 6: A number of 100000; 7: A number of 10000; 8: A number of 1000; 9: A number of 100; 10: Negative control (merely containing IC).

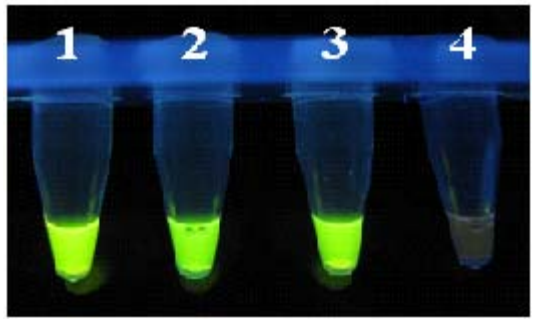

Fig. 3. The optimized LAMP test.
Tubes 1-3: Positive reaction with three strains of *Cryptococcus neoformans*;
Tube 4: Negative reaction

culturing-based methods are still considered as a gold standard in some cases, but in case of need to rapid identification of microorganisms, including *C. neoformans* they generally doesn't satisfy characteristics of an optimal method. False negative results in culture and staining methods ,which need living cells, is due to their low sensitivity and sometimes the samples may be received late to the laboratory, so cells may have been destroyed. Time detection of these yeasts in patients with neuro-cryptococcosis plays an important role in reduced mortality rate from this disease. In Iran and other developing countries the low rate isolation of *C. neoformans*, compared

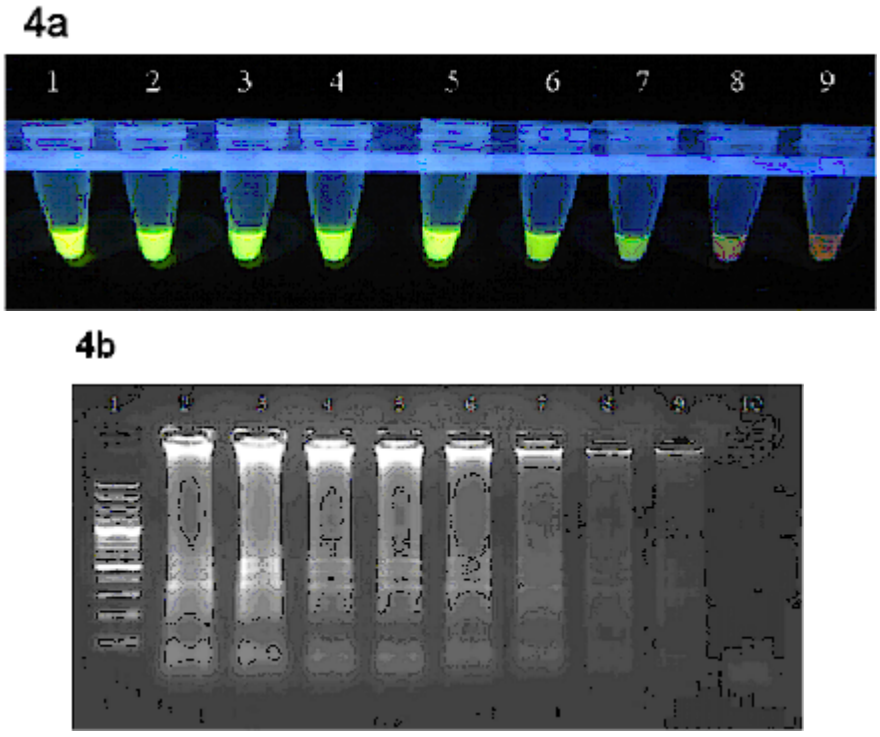

Fig. 4. Determining the sensitivity of LAMP test
4a. Tubes 1: positive control; 2: 1/000/000 cells; 3: 100/000 cells; 4: 10/000 cells; 5:1000 cells; 6: 100 cells; 7: 50 cells; 8: 5 cells; 9: Negative control.
4b. Lines 1: 100 bp DNA Ladder Plus (Fermentas) size marker; 2: Positive control; 3: 1/000/000 cells; 4: 100/000 cells; 5: 10/000 cells; 6:1000 cells; 7: 100 cells; 8: 50 cells; 9: 5 cells; 10: Negative control.

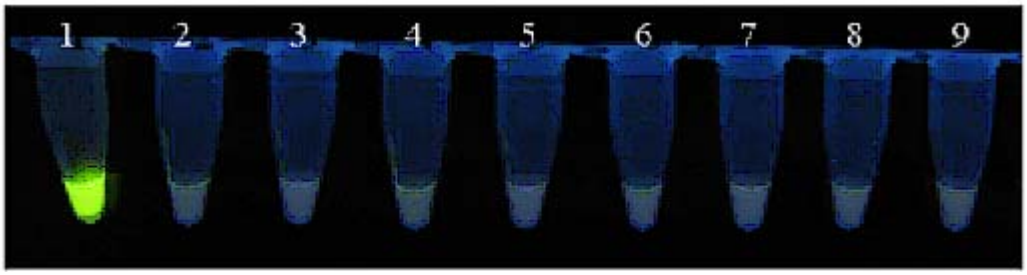

Fig. 5. Determining the specificity of LAMP test
Tube No. 1: positive control; Tube No. 2: LAMP reaction with human DNA; Tube No. 3: With *Candida albicans* DNA; Tube No. 4: With *E. coli* DNA; Tube No. 5: With *Saccharomyces cerevisiae* DNA; Tube No. 6: With *Aspergillus niger* DNA; Tube No. 7: With *Fusarium solani* DNA; Tube No. 8: With *Pichia pastoris* DNA; Tube No. 9: Negative control

to other countries does not indicate low prevalence of infections caused by this yeast, but the difference is related to identification and diagnosis methods. However, studies have shown higher proportion of *C. neoformans* will be detected using much more accurate and sensitive detection methods.

To identify *C. neoformans*, several methods have been already suggested, each one has some advantages and disadvantages. Studies using cultivation techniques along with molecular methods such as the PCR and LAMP methods have shown the culturing method is time-consuming and appears high false negative results. Also, cultures' result will be reported as negative in cases which treatment is done before sampling. So, culturing method results insufficient data in *C. neoformans* detection and application of molecular methods considered as the first priority in diagnosis the agent[8]. Further, more sensitivity and specificity of molecular methods clearly proved by several studies compared serological with molecular methods, such as PCR to detect *C. neoformans*,[25].

In 1984, Cohen, reported that occurrence percentage of false negative results is more than false positive results by comparing diagnostic methods of "Indian Ink" staining, "Acridine Orange" fluorescent staining and serologic Latex agglutination method on 162 *C. neoformans* suspected samples[26]. Velegraki et al. 2001, concluded PCR test on ura5 gene followed by performing the RFLP technique using an "In House" or commercial DNA extraction method can be a good strategy to fast detect of different varieties of cryptococcus by using PCR technique and applying specific primers of *C. neoformans* ura5 gene,[27].

In 2009, Klein et al. reviewed Glycine consuming test results on the CGB Agar medium (l-canavanine glycine bromothymol blue agar) and urea hydrolysis test and melanin production on 102 isolated samples of *C. neoformans* var. *gattii* simultaneously and compared them to results obtained by the "D2 LSU DNA Sequencing" method (by using the "rDNA" of ribosomal large subunit). They declared application of CGB Agar medium with urea hydrolysis test and melanin production on Niger seed Agar can provide good results to distinguish two mentioned species just like as the "D2 LSU DNA Sequencing" method[28].

To extract this agent DNA, various methods have been used in different studies including commercial kits such as QIAGEN[29], Breeden Lab[23], using lysis buffer[30], and freezing / boiling method[31].

There is a little Study which compares different methods of DNA extraction of *C. neoformans* in molecular diagnostics. In present study, the DNP kit was used for clinical samples obtaining good results.

Various parts of the *C. neoformans* genome such as 16S rRNA gene[14], polysaccharide capsule gene[15] and phospholipase enzyme gene[16] incorporated in molecular methods such as PCR under different protocols and techniques to detect the agent. The 16S rRNA gene of genome used by PCR and the ura5 gene were used in the LAMP technique as well because of specificity and constant sequence of that region to detect the agent. In molecular typing of *C. neoformans* and identifying their varieties the followings were used (24).

Finally, sufficiency of ITS1 and CN4 primers to detect *C. neoformans* in cerebrospinal fluid samples as well as, the designed LAMP primers were proved. The optimized conditions of this study led to produce a specific product with high degree of specificity and sensitivity and no unwanted by-products. Although the number of positive samples in this study was not significant, but the existence of cryptococcus or at least its genome in the tested CSF samples appeared in PCR and LAMP tests, which the results were negative in direct microscopic and culturing experiments. The study results also confirmed this issue is true. Although only four positive samples in PCR test and five positive samples in the LAMP test were obtained, but we did not also succeed to identify *C. neoformans* in any of the CSF samples using the available culturing and staining methods. In another similar researches which staining, culturing and PCR methods conducted to detect the yeast, the number of positive samples in PCR were further than culturing and staining methods, which indicates more sensitivity and accuracy of PCR comparing the culturing and staining methods (32).

This research suggests LAMP technique is sufficient. First time in 2000, the LAMP technique

was introduced by a Japanese scientist, Notomi, and his colleagues. Since this technique is able to detect the target sequence in the 6 separated regions by 4 specific primers of FIP, BIP, F3, B3, it acts so specifically in target sequencing. Also, there are more advantages of this method compared to other molecular techniques such as PCR, NASBA, 3SR and SDA (19). Nagamin in 2002, added two primers called loop primers (FLP and BLP) to the reaction and thus the synthesis of DNA in this reaction became faster (18). In 2004, the Japanese scientist, Ushikubo, stated that adding two loop primers of FLP and BLP to the LAMP technique can reduce the required time of the test from one hour to less than half an hour, and also, a great amount of white precipitate of pyrophosphate magnesium is produced as the accessory product in the experiment, which even makes the diagnosis of a positive test possible visually (34). Isothermal replication using a loop is a particular gene duplication method, which DNA is duplicated just by an enzyme with DNA strand displacement feature (such as Bst polymerase) under isothermal conditions (19,33). The frontal inner primer (FIP) includes F2 fragments and the F1 complementary sequence (F1c), and the back inner primer (BIP) includes B2 fragments and the B1 complementary sequence (B1c) in this study, which sometimes allocates larger space sequences of thymidine between these dual fragments that are not necessary either (21,35); F3 and B3 are paired with the initial pattern strand, and with the help of enzyme property to pull the strand, a two-strand is completed and extracted, and simultaneously the strand produced by F1c and B1c will remain as a single-strand for the successive cycles of F1c, B1c, LB, LF performance (19,42). Simply, we could see the LAMP test result by the naked eye. So, this observation is obtainable by using two methods, whether through creating turbidity by addition of cationic polymers or through the addition of SYBR Green fluorescent dye to the LAMP products (20,21). It was proved in this study to detect the opportunistic yeast in clinical samples such as CSFs suspected to neurocryptococcosis, the LAMP technique is more sensitive than the PCR technique. Meanwhile, low cost of this technique regarding required hardware and materials, introduced it as a good alternative for PCR. In several studies other agents such as B and C hepatitis (36, 37), *Mycobacterium tuberculosis* (38) and many other agents were detected, and capabilities of the LAMP technique comparing other molecular methods, was proved, especially in diagnostic purposes.

The study results clearly indicate PCR assay based on 16S rRNA gene sequences and the LAMP test based on ura5 gene are both valuable and reliable techniques for *C. neoformans* detection in cerebrospinal fluid. However, the LAMP technique is a more available method with additional advantages such as higher sensitivity and specificity has no need to expensive hardware and eliminating the identification stage by electrophoresis methods, especially in developing countries.

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