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Full Length Research Paper

Comparison of polymerase chain reaction (PCR) and loop-mediated isothermal amplification (LAMP) for diagnosis of *Fusarium solani* in human immunodeficiency virus (HIV) positive patients

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***Fusarium solani* is the most virulent *Fusarium* sp., frequently reported in the fatal disseminated fusariosis in immunocompromised patients. However, it has been always considered, as a very rare or case report infection among HIV positive patients. Anyhow, because of its irreparable consequences, early diagnosis is very important. In this study, loop-mediated isothermal amplification (LAMP) method was developed for rapid and specific detection of *F. solani* in serum samples of human immunodeficiency virus (HIV) positive patients. Transcription elongation factor (TEF-1α) region was considered as the target gene. The test was carried out in 1 h reaction at 65°C in a heater block. The specificity of the test was 100% and its sensitivity was a copy of genome. Using this method among 45 DNAs samples extracted from HIV positive patients' serums, 9 (20%) cases were positive for *F. solani.* All of the samples were rechecked by polymerase chain reaction (PCR) and the results were the same. Considering these results, it was concluded that due to advantages of the LAMP technique, it can be a better alternative for PCR, even in low technology laboratories. In addition, these findings revealed that the possibility of fatal fusariosis due to *F. solani* is not so rare in HIV positive patients.**

Key words: *Fusarium solani,* loop-mediated isothermal amplification (LAMP), HIV, polymerase chain reaction (PCR).

INTRODUCTION

Fusarium species are common saprophytic fungi that cause a broad spectrum of superficial and systemic infections in human. Systemic or disseminated infection, occurred exclusively in severely immunocompromised

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Table 1. The features of primers.

Name of primer	Sequence of primer
F3fso	5'-GCTTCTCCCGAGTCCCAA-3'
B3fso	5'-AGGAACCCTTACCGAGCT-3'
FIPfso	5'-CTTTGTCCAACGTCGCCCGAGTTTTGCGGTTCGACCGTAAT-3'
BIPfso	5'-AACACCAAACCCTCTTGGCGCAGCGGCTTCCTATTGTTGAA-3'
Lbfso	5'-GCATCACGTGGTTCATAACAGACA-3'
Lffso	5'-GGGGTAAATGCCCCACCAAAAA-3'

patients (Jain et al., 2011; Nucci and Anaissie, 2002). *Fusarium solani* being one of the most virulent species is associated with disseminated disease more frequently than other *Fusarium* sp. The mortality rate of this fusariosis is almost 100%. It can penetrate into the body following a simple onychomycoses or even from the hospital environment (Jain et al., 2011; Nucci and Anaissie, 2007; Lodato et al., 2006; Guarro et al., 2000; Nucci et al., 2003; Ashwini et al., 2013; Galimberti et al., 2012). Invasive infections occur in immunocompromised patients, mainly in association with prolonged and profound neutropenia or severe T-cell immunodeficiency. However, for unknown reasons, this infection has been always considered to be rare among HIV positive patients (Ashwini et al., 2013; Galimberti et al., 2012). It must be mentioned that because of the limitation of diagnostic method the real incidence of these infections is not clear (Galimberti et al., 2012; Azor et al., 2008).

Early diagnosis of fusariosis is important because of its high level of resistance to several antifungal drugs, and its response often requires combination therapy (Ashwini et al., 2013). The utility of classical or conventional methods, however, is limited, as most of the invasive fungal infections are proven only at autopsy. Furthermore, culture or histopathological analysis are usually time consuming and serological techniques are not specific enough to identify the species (Ashwini et al., 2013; Lewis et al., 2006).

Molecular methods based on nucleic acid sequencing, especially gold standard PCR method, are powerful tools for diagnosis of fungal infections and specific identification of etiological agent. However, because of the expensive equipments, like thermal cycler, needed in gold standard method, it cannot be used in a low-technology laboratory. Loop-mediated isothermal amplification (LAMP) is one of the molecular techniques, which was first designed by Notomi and his colleagues in 2000. This method employs a set of six primers that can recognize a total of eight distinct sequences on the target DNA. They are named FIP and BIP as inner primers, B3, F3 as external primers, BLP and FLP as the loop primers. In this method, strand displacement DNA polymerase in isothermal conditions (approximately 65°C) eliminates the need for a thermal cycler. The cycling reaction continues with accumulation of 10^9 copies of target in less than an hour (Mori et al., 2006; Nagamine et al., 2002). Currently, LAMP is mainly applied in the fields of medicine, virus detection, food safety testing and so forth, with less application in detection of fungi, bacteria, nematode and insects (Fukuta et al., 2004; Guan et al., 2010; Jing et al. 2013; Nemoto et al., 2009; Tsujimoto et al., 2007; Nie et al., 2005).

In the present study, LAMP method is established for rapid and specific diagnosis of *F. solani* in serum of HIV positive patients. The results were compared with the results obtained from PCR reference method through chi-square test.

MATERIALS AND METHODS

Extraction of DNA from standard strain

The standard lyophilized *F. solani* strain belonging to bacterial and fungal collection of Iranian Research Organization for Science and Technology (IROST), *PTCC* No. 5284 (UMAH 7419), was cultured in Sabouraud dextrose broth (SDB). After one week incubation, 500 µl of the medium was taken and centrifuged at 10,000 rpm for 2 min.

The supernatant was discarded and the sediment was suspended in 100 µl double distilled water (DDW). Afterwards, DNA was extracted by DNG-plus method. Besides reference strain PTCC NO. 5284 (UMAH 7419), 10 other references strains belong to the collection of TEHRAN university had been used, to be more sure about the accuracy of the obtained results.

Designation of specific primers for LAMP technique

Primers were designed by Primer explorer V4 based on transcription elongation factor (TEF-1α) region of *F. solani* genome (Table 1). The sequences between 29736 to 30046 from mitochondrial genome of the organism were selected and 62978 bps were amplified. The ACC number is JNO 41209.

Reaction mixture for LAMP

LAMP reaction mixture was prepared as following: DDW 5.2 µl, Betaine 5 Mol 4 µl, dNTP (10 mM) 3.5 µl, 10× buffer 2.5 µl, $MgSO_4$ (100 mM) 1.8 µl, primer Mix (A) 1 µl, primer Mix (B) 1 µl, Bst DNA polymerase enzyme (New England BioLabs; Lot:33/110806) 1 µl, target DNA (extracted DNA from standard strain) 5 µl, and total

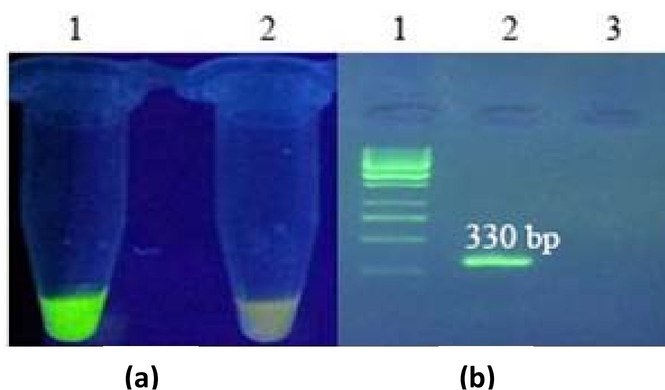

Figure 1. **(a)** LAMP optimization, **(b)** PCR optimization. 1: Positive reaction 1: Size marker 1 kb termoscientific 2: Negative reaction 2: Positive control (330 bp). 3: Negative control.

volume is 25 µl. Primer Mix(A) containing FIP, BIP primers concentration are 40 and 10 µl DDW in 100 µl total volume, respectively, and primer Mix (B) containing LF, LB concentration are 20 and 60 µl DDW in 100 µl total volume, respectively. The reaction was followed by incubation at 65°C for 1 h.

Analysis of LAMP product

At the end of the reaction, 1 µl of 0.1% SYBER Green (*In vitro* gen) was added to each tube. Afterwards the tubes were visualized under UV light.

Identification of LAMP sensitivity and specificity

To determine the sensitivity of the test, different dilution of suspension agent with specified numbers was obtained to study paired primers sensitivity. DNA extraction was done by these dilutions using Boiling with DNG-plus. To provide serial dilution, after measuring the optical density (OD) of extracted DNA from *F. solani*, Genome Copy Number (GCN) was calculated for it. Then a serial dilution of fungal DNA from 1,000,000 copies of DNA (10^{-1} dilution) to 1 copy of DNA (10^{-6} dilution) was prepared. This serial dilution was used for both PCR and LAMP methods, and their sensitivity was compared. DNA extraction was done by these dilutions using Boiling with DNG-plus.

To provide serial dilution, after measuring OD of extracted DNA from *F. solani*, genome copy number (GCN) was calculated for it. Then a serial dilution of fungal DNA from 1,000,000 copies of DNA (10^{-1} dilution) to 1 copy of DNA (10^{-6} dilution) was prepared. This serial dilution was used for both PCR and LAMP methods, and their sensitivity was compared.

DNA of *HSV, Streptococcus pneumoniae, Staphylococcus aureus* and some other species of *Fusarium* except for *F. solani* including F. *oxysporum, F. verticillioides, F. poea, F. graminearum* and *F. prolifratum* were used for specificity test.

Reaction mixture for PCR

The *mtcytb* gene was considered as a target gene for PCR, and ffuso1 (5'-CTC TGT TAA TAA TGC AAC TC-3') and rfuso2 (5'-TGG TAC TAT AGC TGG AGG A-3') were used as specific PCR primers (Dan et al., 2011). PCR was carried out in a total 25 µl reaction mixture containing 5 µl DNA sample, 2.5 µl PCR 10x buffer, 1 µl of forward and reverse specific primer 10 mM, 0.75 µl $MgCl_2$ (50 mM), 0.5 µl dNTP10 mM, 0.4 µl Taq DNA polymerase 5 u/µl followed by primary denaturation at 94°C for 2 min, cycles denaturation at 94°C for 1 min, polymerization at 72°C for 2 min, and final polymerization at 72°C for 10 min. The total number of cycles was 35. The sensitivity and specificity of the test were evaluated by the same method described for LAMP technique.

Analysis of PCR product

The electrophoresis of the reaction product was carried on the agarose gel containing 1.5% SYBER Green (Sina Colon Cat. No.MR7730C) in TBE 0.5 x buffer.

Clinical sample collection and extraction of samples DNAs

A total of 45 serum samples from blood of HIV positive patients (35 samples belonging to Pasture Institute in Tehran and 10 samples belonging to Iranian Research Center for HIV/AIDS (IRCHA), Tehran University of Medical Sciences, and Tehran, Iran) were collected. For safety reasons, firstly, they were inactivated by heating and then their DNA was extracted through DNG-plus method.

Application of optimized LAMP and PCR test for the clinical samples

Both the optimized LAMP and PCR tests were conducted using the entire 45 DNAs, and the results were compared by chi-square test.

RESULTS

At the end of the LAMP reaction, after adding 5 µl SYBR Green to each tube, the positive reaction tube was demonstrated by a bright green fluorescence under UV 366 nm light, while in negative control and negative reaction tubes no fluorescence was observed under UV light (Figure 1a). In parallel, the PCR products were confirmed by electrophoresis on 1.5% agarose jel, the size of PCR product was 330 bp and the target gene was *mtcytb* (Figure 1b). In addition, the results approved by all other 10 mentioned references too.

The results of specificity test indicated that there was no cross reactivity in LAMP technique and its specificity for *F. solani* detection was 100% (Figure 2a). The same results were obtained by PCR method (Figure 2b). In online research with nblast, this data were approved. The detection DNA limit of LAMP technique is one copy of genome. The used PCR technique had the same level of sensitivity and specificity. Using the LAMP method for 45 clinical samples, 9 cases (20%) of them were found to be positive for *F. solani* and 36 cases of them were found to be negative (Figure 3a). The same results were obtained for the same samples by PCR test (Figure 3b). Comparison of the results obtained from two methods by

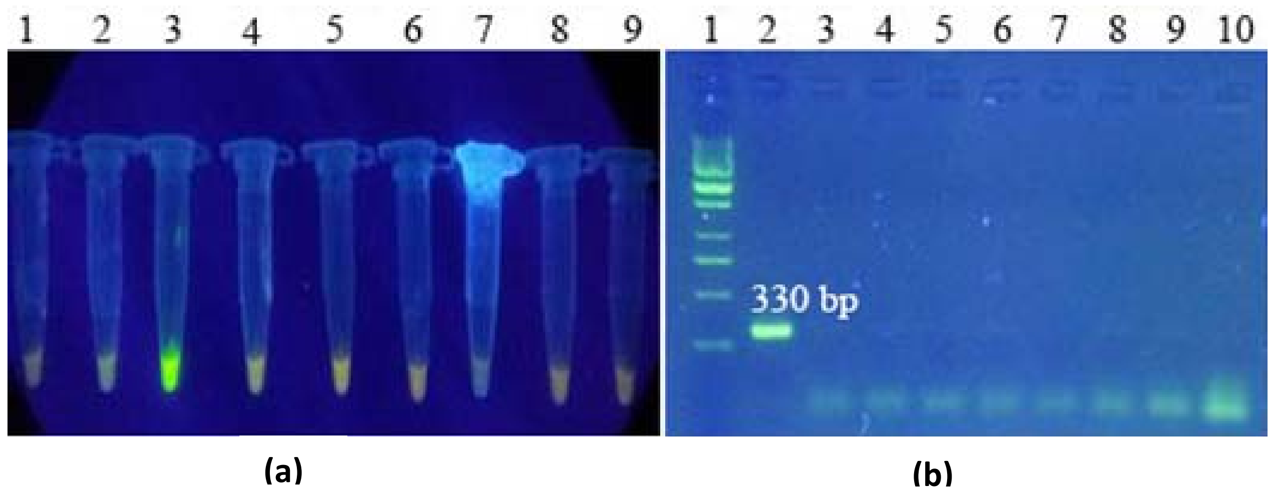

Figure 2. (a): 1: *Streptococcus pneumoniae* (**b):** 1: Middle Range DNA Ladder 1113 Termoscientific. 2: *HSV* 2: *F. solani*; 3: *F. solani* 3: *HSV*; 4: *Staphylococcus aureus* 4: *Streptococcus pneumoniae*; 5: *F. oxysporum* 5: *Staphylococcus aureus*; 6: *F. verticillioides* 6: *F. oxysporum.* 7: *F. poea* 7: *F. verticillioides*; 8: *F. graminearum* 8: *F. poea*; 9: *F. prolifratum* 9: *F. graminearum* ; 10: *F. prolifratum.*

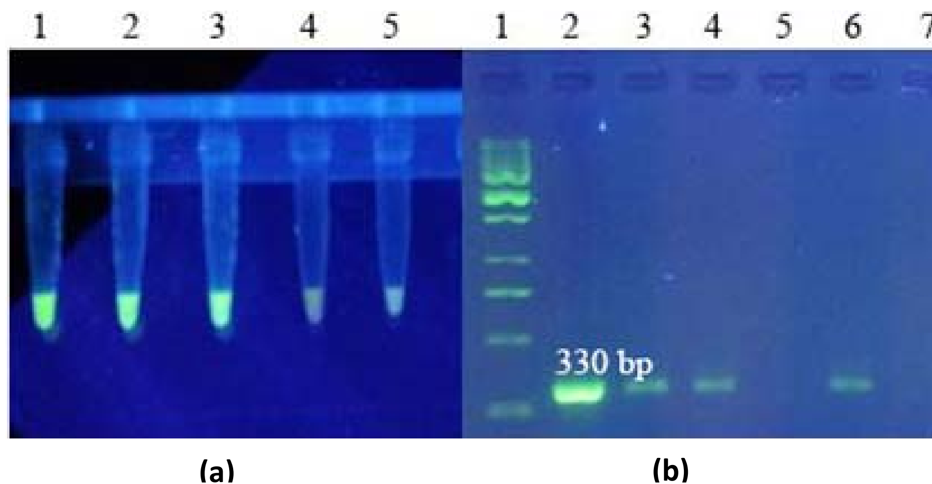

Figure 3. (a): 1: Positive control, (**b):** 1: Size marker DNA Ladder 1 Kb Termoscientific. 2 and 3: Positive sample; 3, 4 and 6: positive sample; 4: Negative sample; 5: Negative sample; 5: Negative control; 7: Negative control.

Chi-squared test showed that both techniques are equally for diagnosis of *F. solani* in serum of HIV positive patients.

DISCUSSION

Fusariosis as the second most frequent systemic mycosis is associated with high mortality (Jain et al., 2011; Lodato et al., 2006) and *F. solani* as the most virulent species, is also associated with disseminated disease more frequently than other *Fusarium* sp. (Jain et al., 2011). Successful treatment is linked to early diagnosis of this infection (Guarro et al., 2000; Galimberti et al., 2012). Identification of species level is highly important because different species have different antifungal sensitivity (Alcazar et al., 2008; Tortorano et al., 2008; Lo¨effler et al., 2002; Lo¨effler et al., 1997). This is particularly more vital when two different species cause infection at the same time. Guarro et al. (2000) reported a case of mixed infection caused by two species of *Fusarium* in a human immunodeficiency virus-positive patient with lymphoma who was neutropenic due to chemotherapy. The patient showed the typical signs of a disseminated fusarial

infection, with *F. solani* isolated from skin lesions and *F. verticillioides* isolated from blood. The report discusses how difficult it is to make an accurate diagnosis when an immunosuppressed patient is infected with more than one fungal species, especially when the species are morphologically very similar (Guarro et al., 2000).

LAMP method is a very powerful tool for identifying the species, particularly, in a mix infection. Due to the use of six primers for target gene, this technique is highly specific in identifying the species. Some important factors including efficacy of DNA extraction and quality of designed primers will affect the sensitivity and specificity of the assay (Lo¨effler et al., 1997; Einsele et al., 1997; Francois et al., 1999). In order to use molecular methods like RAPD, RFLP and PCR for isolation of *Fusarium*, the DNAs were previously developed (Godoy et al., 2004; Thomas et al., 2003; Oriel et al., 2005). The major objective of the current study was to develop the LAMP method and further optimize it for specific, sensitive and rapid detection of organism directly in sample, even in low technology laboratory. In this study, LAMP test was carried out in 1 h; however, for the same analysis by PCR method, the passed time was 3 h. Apart from rapidity, we observed an equal level of sensitivity and specificity using the LAMP method compared to gold standard PCR test. However, in some other studies, a higher level of sensitivity was reported for the LAMP method as compared to the PCR method (Kuboki et al., 2003; Bakheit et al., 2008; O'Donnell et al., 2007; Helm et al., 1990). In general, LAMP offers a better alternative, and the major advantages of this method are possibility of visual judgment by color, being time saving, and not requiring the costly PCR apparatus and gel scanner.

As it is mentioned above, quality of designed primers and the sequences of elected target genes can affect the sensitivity and specificity of the molecular assays (Jain et al., 2011; Lewis et al., 2006; Kuboki et al., 2003). Different molecular studies employ richly varied sequences of multiple loci such as elongation factor 1α (EF-1α)), β-tubulin (β-TUB), calmodulin (CAM), RNA polymerase II second largest subunit (RPB2), the nuclear ribosomal Internal Transcribed Spacer (ITS) region, domains D1 and D2 of the 28S ribosomal DNA (rDNA) large subunit for *Fusarium* sp. identification. However, the available data clearly demonstrate that sequences from the (ITS) region and domains D1 and D2 of the 28S ribosomal DNA (rDNA) large subunit are too conserved to resolve most of the clinically important fusaria at the species level. Moreover, use of ITS and β-tubulin within *F. solani* species complex should be avoided due to paralogous or duplicated divergent alleles (Jain et al., 2011; Guarro et al., 2003; Arif et al., 2012; Eljaschewitsch et al., 1996). In this study, TEF-1α was considered as the target gene. This sequence, according to the reported results (Arif et al., 2012) was highly specific for *F. solani* and did not show cross reaction with any other similar species of *Fusarium* (Eljaschewitsch et al., 1996). This agreed with the obtained results in the current study. As it has been shown in Figure 2a, the specificity of the test is 100%. In online research with nblast, this data was approved. Therefore, the established test can precisely detect *F. solani* in the sample and differentiate the organism even in a mixed infection. Moreover, as it can detect even one copy of genome in the sample, this technique can be very effective for rapid and accurate diagnosis of *F. solani* in serum of HIV positive patients at the early stages of infections.

In the present research, the total number of samples was 45, and nine cases (20%) of them were detected to be positive for *F. solani* by both PCR and LAMP methods. As previously described, for unknown reasons this infection has been always considered to be rare in the population of HIV positive patients (Ashwini et al., 2013; Muhammed et al., 2011). A review of 294 patients published in 2005, identified only two cases of disseminated *Fusarium* infection in HIV positive patients (Perfect, 2012) and no cases of disseminated *Fusarium* infection have been reported since 2005 (Galimberti et al., 2012).

In April 2013, a case report of fatal disseminated fusariosis due to *F. solani* in a HIV positive patient was published. This case represented the first report of fatal disseminated *Fusarium* infection in a non-neutropenic HIV positive patient (Ashwini et al., 2013). As it has been mentioned, although fusariosis is the second responsible organism for disseminated fusariosis, but this infection has been always considered as a rare infection in HIV positive patients. Ashwini et al. (2013) claimed that the two most significant predisposing risk factors for disseminated filamentous fungal infections are severing prolonged neutopenia and T cell deficiency. The first one is less common in HIV patients and the second one has also become less common because of highly active antiretroviral therapy (HAART), at the asymptomatic stage of disease. The mentioned report concluded that because of these reasons this infection is rare in this population. This can be true; however, it seems that due to limitation of diagnostic methods, the real incidence of these infections is unknown. Since they are poorly detected, laboratories and clinical microbiologists are not generally aware of their possible presence in human infections (Azor et al., 2008). Sometimes just after appearing disseminated lesions on the skin of patient, or after positive blood culture this can be identified as Fusariosis, however; in some cases like patient in the article, there is no lesion on the skin of patient. Therefore it causes unawareness of the presence of organism in the body of the host. It seems that, the number of hidden infections must be more than what has been reported. On the other hand because of using corticostoid to prevent, (IRIS) syndrome in these patients, the risk of IFI is increased. Maybe in the first stages of the disease,

because of HAART, it cannot be so obvious, but gradually after decreasing the number of T cell, progressively, it will appeared.

However, proof of the presence of organism in the serum sample does not mean that there is infection at the moment, but it shows that the potentiality of infection is not so rare but maybe it has been ignored or has not been detected clearly by now, but by this developed method it could be distinguished very soon and this can be useful for prevention of disseminated infection.

The obtained results are very highly reliable, because the same samples have been rechecked by gold standard PCR, and the positive and negative controls have been always used in each group of samples.

Generally, early diagnosis is important to manage the consequences of infections. For instance, the results of different researches have shown increased survival of patients by combination therapy (Perfect, 2012; Ho et al., 2007; Vagace et al., 2007). LAMP, as a more recent molecular technique for diagnosis, has some advantages like rapidity, sensitivity, cost effectiveness and easy visual result judgment. To our knowledge, this is the first attempt to establish this technique for detection of *F. solani* in serum of HIV positive patients. The results were compared with the results of gold standard PCR method by chi-squared test. As the results show, both methods to the last dilution of DNA (10^{-6} = 1 copy of DNA) were positive. In addition, using both of these techniques, for clinical samples, the same specimens had the same results. Considering these findings, it can be concluded that both tests had the same level of sensitivity and were equal for diagnosis of *F. solani* in serum samples. Besides the general advantages which were mentioned previously for LAMP techniques, the high level of specificity and sensitivity of this optimized test makes it proper for precise identification of pathogen at early stage of disease and it can be used in all clinical laboratories instead of PCR. It suggests that this method can be applied for other high risk groups for disseminated infection like patients with hematologic malignancies in cytotoxic therapy and transplant recipient.

Conclusion

According to the results obtained in this research, it can be concluded that because of accuracy, rapidity and its cost effectiveness, the LAMP technique can be a better alternative for gold standard PCR, even in low technology laboratories. In addition, the results imply that this fatal infection should no longer be considered so rare in HIV positive patients.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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