Strategies for Isothermal Amplification of Nucleic Acids: are they Ready to be Applied in Point of Care Diagnosis of Mycosis?

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Abstract: The early detection of invasive fungal infection (IFD) is significant in order to decrease mortality in susceptible patients. There is, therefore, a need for sensitive and specific fungal species detection assays in a clinical laboratory for early targeted therapy. The isothermal amplification method may be useful for the screening of fungal isolates, especially in resource-poor settings. Therefore, our aim was to review the isothermal nucleic acid amplification methods and their applications in fungal pathogen detection. Out of 50 reported studies, 28, 12, 6, 2, and 2 studies used the isothermal-based assays of a loop-mediated isothermal amplification (LAMP), nucleic acid sequence-based amplification (NASBA), rolling circle amplification (RCA), multiple displacement amplification (MDA) and polymerase Spiral Reaction (PSR), respectively. Thirty-two studies used clinical samples, 18 pure culture, and four environmental samples. The diagnostic accuracy of isothermal nucleic acid amplification testing for pathogenic fungal was reported as high (sensitivity 0.89–1.0 and specificity 0.63–1.0) in all studies irrespective of the sample tested. Although the isothermal-based assays showed high sensitivity and specificity in reported studies, it is still poorer than that of PCR assays. However, improving the assay to make it simpler, more effective, and inexpensive compared with newer PCR methods are still needed.

Keywords: Isothermal Molecular Diagnostics; Invasive fungal infection; Precision testing; Simple detection.

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1. Introduction

Fungi are ubiquitous microorganisms, which increasingly are recognized as emerging opportunistic pathogens of clinical relevance due to their high morbidity and mortality infection rate among immunocompromised individuals and other highly susceptible patients [1, 2]. Invasive fungal diseases (IFD) [3] are one of the major causes of morbidity and mortality among populations at high risk, including those with corticosteroid therapy, uncontrolled diabetes, solid organ or allogeneic stem cell transplant recipients, and patients undergoing immunosuppressive therapies [2, 4]. The global reported incidence of chronic pulmonary
aspergillosis is 3,000,000 cases; invasive aspergillosis is ~250,000 cases; invasive candidiasis is ~700,000 cases; and cryptococcal meningitis in human immunodeficiency virus (HIV)-infected patients is ~223,100 cases annually [3, 5, 6]. The gold standard for diagnosing IFD is based on histopathological and mycological findings, followed by radiologic manifestations [3]. The patients suspected of IFD should be immediately treated in order to improve the clinical outcomes [7]. Despite our improved understanding of IFD and the availability of new antifungal treatment, the survival rate in IFD remains poor [8, 9]. The detection of the IFD case in the early days of infections is critical for minimizing the mortality rate in susceptible patients [10, 11]. There is, therefore, an urgent need for sensitive and specific fungal species detection assays in a clinical laboratory for early targeted therapy. Molecular diagnostics have shown advantages in the clinical diagnostic laboratory for routine detection, epidemiologic, and fingerprinting analysis of fungal infections [2, 12-14]. Molecular diagnostics reduce the time required for the morphology and biochemistry identification, including the selection of fungal culture media and the incubation period [2, 12]. This diagnostic method can be used to detect fungal isolates directly from clinical specimens [15], which reduces the exposure to the possible infection agents present in the patient samples. Current development in a polymerase chain reaction (PCR) has led to the advancement of newer molecular amplification methods which isothermal amplification methods are considered one of the most important classes of molecular diagnostic methods [16]. Considering the successful applications of isothermal amplifications in many fields of diagnostics, isothermal amplification methods may be useful for screening of fungal isolates, especially in resource-poor settings or for point-of-care testing [17]. Therefore, our aim was to review the current state of the art in isothermal nucleic acid amplification methods and their applications in fungal pathogen detection.

2. Materials and Methods

2.1. Study design.

The review process involved the study of existing published literature of all reported published papers based on isothermal nucleic acid amplification identification of pathogenic fungi until January 2020.

2.2. Search strategy.

To search the published literature, Medline database through PubMed, Embase through Scopus, ISI Web of Science, Science Direct, and Google Scholar were used to retrieve the full-text of articles of isothermal nucleic acid amplification identification of pathogenic fungi using the key words “DNA amplification”, “polymerase chain reaction” or “PCR”, “isothermal nucleic acid amplification”, “loop-mediated isothermal amplification” or “LAMP”, “nucleic acid sequence-based amplification” or “NASBA”, “rolling circle amplification” or “RCA”, “helicase-dependent amplification” or “HDA”, “strand displacement amplification” or “SDA”, “multiple displacement amplification” or “MDA” and “polymerase Spiral Reaction” or “PSR”, “fungal detection method” or “molecular diagnosis”, “fungal infection”, “fungal”, “yeast”, “Candida albicans”, “Aspergillus fumigatus” and “pathogenic fungi” in different combinations. A total of 52 relevant articles were returned using these keywords. All references were compiled into a database and managed using EndNote Library version X9.

2.3. Data extraction.
The data was organized in master sheets, which were separated for each paper. The following data items were collected: amplification method, samples, genus and species of fungal, detection method, assay type, target, test duration, limitation of detection, and specificity of each method.

3. Results and Discussion

We retrieved 123 published articles, which yielded 52 papers included in this review. Ten, nine, and six were from Japan, China, and Germany, respectively; three were from each of Brazil, The Netherlands, Korea, Australia, and Iran; two were from each of Portugal, USA, and France, and one was from each of Italy, UK, New Zealand, and India. Among the 52 studies, 36 used the cohort approach, five used the case-control approach, and nine did not describe the recruitment approach. Analysis of the total 52 papers included in this review was done based on the following aspects: amplification method, samples, genus and species of fungal, detection method, assay type, target, test duration, limitation of detection, and specificity of each method. (Table 1) [18-70]. Thirty-three studies used clinical samples, 19 pure culture, and four environmental samples. In addition, 29, 12, 7, 3, and 2 studies used the isothermal-based assays of LAMP, NASBA, RCA, MDA, and PSR, respectively. The diagnostic accuracy of isothermal nucleic acid amplification testing for pathogenic fungal was reported as high (sensitivity 0.89–1.0 and specificity 0.63–1.0) in all studies irrespective of the sample tested (pure culture, clinical and environmental samples). Two of the studies provided diagnostic accuracy on NASBA testing for Aspergillus and Candida species in blood specimens of patients suffering from invasive aspergillosis and candidiasis [46, 71]. Paracoccidioides brasiliensis, Histoplasma capsulatum, Candida species, Saccharomyces species, Aspergillus species, Trichosporon species, Cryptococcus neoformans, Penicillium marneffei, Pneumocystis jirovecii, Fonsecaea species, and Fusarium species were identified using isothermal amplification-based techniques in these studies. The sensitivity of LAMP testing for Candida auris was reported as high in the Yamamoto et al. [26] investigation (2 × 10^1 copies per reaction). Candida species was the most frequently detected pathogen among blood samples (n = 55), and sensitivity for Candida species detection by NASBA and PSR was 1–100 CFU/ml and 6.9 pg/ml, respectively, with a specificity of 100%. All LAMP reactions were completed within 60 min from start to finish per clinical sample. The most common target genes used in these 50 studies were the ITS gene in 16 studies and 18S rDNA in 13 studies. A comparative study of isothermal-based assays employing primers targeting 18S rRNA and ITS genes revealed a similar limit of detection for both genes.

Although newer DNA amplification methods have the potential to considerably influence the diagnosis of fungal infectious diseases [2], the performance of a molecular assay is generally more expensive than conventional diagnostic laboratory methods. Conventional diagnostic/identification methods require a minimum of 24 h, and in various cases, significantly longer. Some fungal species causing human infections, such as Scedosporium species [72], Malassezia species [73], and Mucorales [74], are not easily detected by routine culture methods and require specialized procedures. In the reported studies, the isothermal amplification-based techniques provided the summary estimate sensitivity of 89–100 % and specificity of 63–100%. Our results were supported by some factors. First, the strength of the current study is that we considered diagnostic assay focusing on isothermal amplification and pathogenic fungal. Second, we carried out an attentive study search and included as many as 50 studies. Third, sensitivity analyses consistently in reported studies revealed similar overall
diagnostic ability among different isothermal-based assays. Alternative isothermal amplification-based methods such as LAMP is available for the detection of fungal pathogens [18]. LAMP is relatively sensitive, specific, and cost-effective [17, 75]. Furthermore, numerous isothermal amplification-based methods have recently emerged in the field of fungal pathogen detection due to their cost-effectiveness and rapidness [2, 15]. The potential benefits of earlier diagnosis methods of an invasive fungal infection include the timely initiation of appropriate antifungal therapy, which may prevent progressive tissue invasion, lead to decreased mortality, and an overall improvement in healthcare utilization and anti-antifungal stewardship [76]. If isothermal amplification testing for the fungal pathogen is to be used as a rapid tool, further studies are required to demonstrate the safety of this approach. The isothermal-based assays combined with conventional methods may be an acceptable diagnostic strategy, especially in resource-poor countries. A combination of several rapid methods for the detection of a particular fungal pathogen can also be possible as the use of only one detection test may not be sufficient to confirm the identified fungal pathogen [77]. The effect of different combinations of rapid methods for fungal human pathogen detection in order to develop the most effective and accurate detection method would be considered. The overall sensitivity of isothermal testing is high, but the existing studies using blood and clinical samples are small. In the future, it should be focused on other clinical sample types, e.g., urine, stool, blood, plasma, serum, CSF, BAL, and throat swabs, to test the clinical application of the fungal species-isothermal amplification assay in diverse samples [78]. In conclusion, we conducted a review study to reveal the use of the isothermal amplification assay in the diagnosis of fungal species causing human infections. Although the isothermal-based assays showed very good sensitivity and specificity in reported studies, they still needed to be improved to become simpler, more effective, and cheaper compared with PCR-based methods.

4. Conclusions

There are a variety of studies to detect fungal infection, and it has to be selected the optimal diagnostic/identification tools according to the prevalence of a fungal infection in the area in patients suspected of IFD. However, cost issues concerning the diagnostic strategy should be considered. Although PCR methods are the best examinations currently available [79-82], isothermal amplification-based techniques may be considered as an alternative test in resource-poor and developing countries. Isothermal amplification testing can be performed in 1 h [70], and it could be used prior to initiation of antifungal therapy. The overall sensitivity of isothermal testing is high, but the existing studies using blood and clinical samples are small. In the future, it should be focused on other clinical sample types, e.g., urine, blood, CSF, BAL, and throat swabs, to test the clinical application of the fungal species-isothermal amplification assay in diverse samples. In conclusion, we conducted a review study to reveal the use of the isothermal amplification assay in the diagnosis of fungal species causing human infections. Although the isothermal-based assays showed very good sensitivity and specificity in reported studies, they still needed to be improved to become simpler, more effective, and inexpensive compared with PCR-based methods, especially for using them in point of care diagnosis.
Table 1. Employed isothermal nucleic acid amplification technologies for the diagnosis of pathogenic fungi.

| No. | Study | Amplification Method | Samples | Fungal species | Read-out method | Assay type | Target gene | Turn-around time | Sensitivity | Specificity |
|-----|-------|----------------------|---------|----------------|----------------|------------|-------------|----------------|-------------|-------------|
| 1   | Endo S., et al. [11] | LAMP | Clinical isolates culture | Paracoccidioides brasiliensis | AGE | LAMP | Gp43 | 60 min | 100 fg DNA/rxn | 100% |
| 2   | Tatiibana B., et al. [2] | LAMP | Human sputa samples | Paracoccidioides brasiliensis | AGE | LAMP | Gp43 | 60 min | 100 fg DNA/rxn | 100% |
| 3   | Trabasso P., et al. [3] | LAMP | Clinical and reference isolates culture | Candida parapsilosis | turbidity | Real-time LAMP | topoisomerase II | 60 min | Not determined | 100% |
| 4   | Noguchi H., et al. [4] | LAMP | Oral exfoliative cytology samples | Candida albicans | turbidimetry | Real-time LAMP | ITS | 60 min | 1 pg DNA/rxn | 100% |
| 5   | Goodarzi M., et al. [5] | LAMP | Bronchoalveolar lavage (BAL) sample culture | Candida albicans | AG, fluorescence | Real-time LAMP | alph-INT1 | 60 min | 10 copies/rxn | 100% |
| 6   | Inacio J., et al. [6] | LAMP | Pure culture mycelium | Candida species | AGE, fluorescent | Hybridization LAMP | D1/D2 | 90 min | 50 fg /rxn | 100% |
| 7   | Kasahara K., et al. [7] | LAMP | Artificial contaminated dairy product | Candida and Trichosporon species | turbidimetry | Real-time Multiplex LAMP | ITS-2, IGS-1 | 60 min | 3.2 × 10^1 - 8.0 × 10^2 cells/mL | 100% |
| 8   | Nakayama T., et al. [8] | LAMP | Environment Swab Specimens | Candida albicans, Cryptococcus neoformans | turbidimetry | Real-time LAMP | LSU rRNA | 60 min | 10-100 copies/rxn | 100% |
| 9   | Yamamoto M., et al. [9] | LAMP | Ear swab specimen PCM | Candida auris | AGE | LAMP | ITS | 60 min | 2 × 10^1 copies/rxn | 100% |
| 10  | Tang Q., et al. [10] | LAMP | PCM | Aspergillus fumigatus | AGE, fluorescence | LAMP | anxC4 | 60 min | 10 copies/rxn | 100% |
| 11  | Storari M., et al. [11] | LAMP | PCM | Aspergillus species | Visual inspection of color change | LAMP | Polyketide synthase | 60 min | Between 0.1 and 0.01 ng/rxn | 100% |
| 12  | Yu L.S., et al. [12] | LAMP | Clinical samples culture | Aspergillus fumigatus | Fluorescence, chlorimetry | Allele-specific LAMP | Cyp51A | 60 min | Down to 10 copies | 100% |
| 13  | Lucas S., et al. [13] | LAMP | PCM | Cryptococcus species | AGE | LAMP | CAP59 | 60 min | 100-500 fg | 100% |
| 14  | Matsuzawa T., et al. [14] | LAMP | PCM | Emericella species | AGE, turbidimetry | Real-time LAMP | Hydrophobin | 60 min | 10 pg | 100% |
| 15  | Scheel C.M., et al. [15] | LAMP | PCM and urine specimens | Histoplasma capsulatum | AGE | LAMP | HCP100 | 60 min | 1 to 30 copies/rxn | 100% |
| No. | Study                                      | Amplification Method | Samples                              | Fungal species                     | Read-out method          | Assay type | Target gene      | Turn-around time | Sensitivity | Specificity |
|-----|-------------------------------------------|----------------------|--------------------------------------|-------------------------------------|--------------------------|------------|------------------|------------------|-------------|-------------|
| 16  | da Silva Zatti M., et al. [16]             | LAMP                 | Bone marrow and whole blood specimen culture | *Histoplasma capsulatum*              | AGE, fluorescence       | LAMP       | ITS-1            | 60 min           | 1 fg/μl     | 100%        |
| 17  | Sun J., et al. [17]                        | LAMP                 | Human biopsy specimens’ culture       | *Penicillium marneffei*              | AGE, fluorescence       | LAMP       | ITS              | 60 min           | 2 copies/rxn | 100%        |
| 18  | Uemura N., et al. [18]                     | LAMP                 | Sputum and BALF samples              | *Pneumocystis jirovecii*             | fluorescence, turbidimetry | Real-time LAMP | 18 S rRNA     | 60 min           | 50 copies/rxn | 100%        |
| 19  | Singh P., et al. [19]                      | LAMP                 | Sputum and BALF samples              | *Pneumocystis jirovecii*             | AGE                      | LAMP       | 18 S rRNA       | 60 min           | 1 pg         | 100%        |
| 20  | Ishikawa H., et al. [20]                   | LAMP                 | PCM                                  | *Filobasidiella neoformans*          | turbidimetry             | Real-time LAMP | ITS-2          | 60 min           | 10^9 cells/mL | 100%        |
| 21  | Zhou J., et al. [21]                       | LAMP                 | PCM and blood samples culture         | *Trichosporon asahii*                | AGE, fluorescence, turbidimetry | Real-time LAMP | IGS             | 60 min           | 3.56 × 10^7 - 3.56 × 10^9 copies/rxn | 100%        |
| 22  | Sun J., et al. [22]                        | LAMP                 | PCM                                  | *Fonsecaea species*                  | AGE, fluorescence, turbidimetry | LAMP       | ITS              | 60 min           | 0.2 fg       | 100%        |
| 23  | Niessen L., et al. [23]                    | LAMP                 | PCM and cereal samples               | *Fusarium tricinctum*                | AGE                      | LAMP       | Tef-1 α and acl1 | 60 min           | 0.95 pg/rxn | 100%        |
| 24  | Niessen L., et al. [24]                    | LAMP                 | PCM                                  | *Fusarium graminearum*               | AGE                      | LAMP       | gaoA             | 60 min           | 2 pg         | 100%        |
| 25  | Denschlag C., et al. [25]                  | LAMP                 | PCM                                  | *Fusarium species*                   | turbidimetry             | Real-time LAMP | tri6 and tri5  | 60 min           | 0.004-1.572 ng | 100%        |
| 26  | Luo J., et al. [26]                        | LAMP                 | PCM                                  | *Aflatoxigenic species*              | fluorescence             | LAMP       | acl1 and amy1    | 60 min           | 2.4 pg -20 pg | 100%        |
| 27  | Luo J., et al. [27]                        | LAMP                 | PCM                                  | *Aflatoxigenic Aspergilli*           | turbidimetry             | Real-time LAMP | acl1 and amy1   | 60 min           | 10-100 spores/reaction | 100%        |
| 28  | Hayashi N., et al. [28]                    | LAMP                 | PCM                                  | *Saccharomyces species*              | AGE, turbidimetry        | Real-time LAMP | Lg-MET16 Sc-MET16 Sb-RAD18 Sp-TLC1 STA1 | 60 min           | 1.9 × 10^1 - 4.4 × 10^3 CFU | 100%        |
| No. | Study | Amplification Method | Samples | Fungal species | Read-out method | Assay type | Target gene | Turn-around time | Sensitivity | Specificity |
|-----|-------|----------------------|---------|----------------|----------------|------------|-------------|----------------|-------------|-------------|
| 29  | Widjioatmodjo M.N., et al. [29] | NASBA | Clinical whole blood samples | Candida species | AGE, enzymatic bead-based detection | NASBA | 18S rRNA | 100 min | 0.01 CFU | 100% |
| 30  | Borst A., et al. [30] | NASBA | Clinical blood culture samples | Candida albicans | ECL | NASBA | 18S rRNA | 100 min | 10–100 CFU/ml | 100% |
| 31  | Loeffler J., et al. [31] | NASBA | Clinical blood culture samples | Candida species | ECL | NASBA | 18S rRNA | 90 min | 1 CFU | 100% |
| 32  | Loeffler J., et al. [32] | NASBA | Clinical blood culture samples | Aspergillus species | ECL | NASBA | 18S rRNA | 90 min | Not determined | 63% |
| 33  | Yoo J.H., et al. [33] | NASBA | whole Blood samples | Aspergillus species | fluorescence | Real-time NASBA | 18S rRNA | 90 min | Not determined | 96% |
| 35  | Zhao Y., et al. [35] | NASBA | BAL fluids | Aspergillus fumigatus | fluorescence | Real-time NASBA | 28S rRNA | 90 min | Not determined | >95% |
| 36  | Zhao Y., et al. [36] | NASBA | PCM | Candida, Aspergillus species | fluorescence | Real-time NASBA-MB | 28S rRNA | 90 min | Not determined | 10^1–10^6 copies |
| 37  | Park C., et al. [37] | NASBA | Blood culture | Aspergillus species | fluorescence | FRET-NASBA | 18S rRNA | 90 min | 6 x 10^6 copies/ml | 100% |
| 38  | Wang L., et al. [38] | NASBA | Plasma samples | Aspergillus species | AGE | NASBA | 18S rRNA | 100 min | Not determined | 80.43% |
| 39  | Brenier-Pinchart M.P., et al. [39] | NASBA | PCM | Filamentous fungi | Fluorescence | Real-time NASBA | 28S rRNA | 90 min | 0.5 spore/NASBA | 100% |
| 40  | Du L., et al. [40] | NASBA | plasma samples | Aspergillus fumigatus | Colorimetry | NASBA-ELISA | 18S rRNA | 100 min | 1 CFU | 100% |
| 41  | Zhou X., et al. [41] | RCA | Clinical isolates culture | Candida, Aspergillus, Scedosporium species | AGE | RCA | ITS2 | 60 min | 10^7 copies/rxn | 100% |
| 42  | Kaocharoen S., et al. [42] | RCA | Clinical specimens' culture | Cryptococcus species | AGE, fluorescence | Real-time Hyper branched RCA | ITS | 60 min | Not determined | 100% |
| No. | Study | Amplification Method | Samples | Fungal species | Read-out method | Assay type | Target gene | Turn-around time | Sensitivity | Specificity |
|-----|-------|----------------------|---------|----------------|----------------|------------|-------------|------------------|-------------|------------|
| 43  | Trilles L., et al. [43] | RCA | Clinical isolates culture | Cryptococcus neoformans and Cryptococcus gattii | AGE, fluorescence | Hyper branched RCA | PLB1 | 70 min | 40 copies/rxn | 100% |
| 44  | Furuie J.L., et al. [44] | RCA | Clinical isolates culture | Histoplasma capsulatum | AGE | RCA | ITS1 | 90 min | 10⁶ DNA copies/rxn | 100% |
| 45  | Kong F., et al. [45] | RCA | Clinical isolates culture | Trichophyton species | fluorescence | RCA | ITS2 | 90 min | 10⁶ copies/rxn | 100% |
| 46  | Najafzadeh M., et al. [46] | RCA | Clinical and environmental samples | Fonsecaea species | AGE | RCA | ITS | 90 min | Not determined | 100% |
| 47  | Hamzehei H., et al. [47] | RCA | Clinical and environmental samples | Cladophialophora species | AGE | RCA | ITS | 90 min | Not determined | 100% |
| 48  | Sun J., et al. [48] | MDA | Clinical isolates culture | Penicillium marneffei | AGE | SNP-RCA | ITS1 and ITS2 | 120 min | 6.025×10⁵/tube | 100% |
| 49  | Zhao F., et al. [49] | MDA | Clinical sputum samples | Candida albicans | AGE, Colorimetry turbidimetry | MCDA-LFB | ITS2 | 40 min | 200 fg | 100% |
| 50  | Foster S., et al. [50] | MDA | PCM | Filamentous fungi | AGE | MDA | WGA | 18 h | 1–10 copies/rxn | 100% |
| 51  | Jiang X., et al. [51] | PSR | Blood samples culture | Candida albicans | turbidimetry, calorimetry | Real-time PSR | ITS2 | 60 min | 6.9 pg/ml | 100% |
| 52  | Sakai K., et al. [52] | RPA | Blood samples culture | Pan-fungal detection | AGE | PSR-microarray system | ITS | 40 min | 10³ CFU/ml | 100% |

LAMP: Loop-mediated isothermal amplification, NASBA: Nucleic acid sequence-based amplification, MDA: Multiple displacement amplification, FRET: Fluorescence resonance energy transfer, AGE: Agarose gel electrophoresis, PCM: pure culture mycelium, BALF: Bronchoalveolar lavage fluid, ECL: Electrochemiluminescence, SNP: Single Nucleotide Polymorphism.
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Conflicts of Interest

The authors declare no conflict of interest.

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