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A Rapid Protocol of DNA Extraction for Molecular and Immunological Identification of *Aspergillus* species

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Abstract

Morphological characterization has gradually been replaced by molecular DNA methods to specifically identify the species of fungi. Therefore, an efficient, fast, and inexpensive procedure to get the fungal genomic DNA is very desirable in a variety of applications, including DNA barcoding and genetic epidemiology. This study was aimed at designing a quick ribosomal DNA (rDNA) extraction from fungal pure cultures of ascomycetes and also validating the specificity of monoclonal antibody JF5 in the recognition of antigens of the genus *Aspergillus*. The development of the DNA extraction protocol was tested on 60 isolates of ascomycete fungi, and the conventional CTAB methodology was used as the control. Universal ITS primers were used to sequence the amplified ITS regions. At the same time, direct ELISA of surface wash antigens of the tested fungi was carried out. ITS sequencing established the great efficiency (100%) of the rapid protocol and resulted in amplifiable DNA of the appropriate size for all tested fungal species. This molecular recognition was confirmed immunologically for *Aspergillus*. JF5 was able to show high specificity toward antigens of the genus *Aspergillus*. The developed protocol for *Aspergillus* identification is simple, harmless, and can process several samples simultaneously. JF5 exhibited high specificity for *Aspergillus* antigen detection.

Keyword: JF5; *Aspergillus*; PCR; ITS; ELISA.

Introduction

Fungi have emerged as opportunistic pathogens associated with severe infections in humans, such as invasive mycoses, particularly in immunocompromised individuals [1]. The species of *Candida* and *Aspergillus* are the most frequent opportunistic pathogens [2]. *Aspergillus* species are considered to have the potential of being pathogenic and being involved in severe infections, causing invasive pulmonary infections, Aspergillosis, and allergic disease[3]. *Aspergillus* conidia are widespread in nature. After inhalation or

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inoculation, an infection may end up locally dispersed or have a far-reaching location depending on the host's immune status [4]. There is a great change in risk factors that predispose an individual to this infection due to the emergence of various biological agents of the immune system and the emergence of viral infections like coronavirus disease [5]. Although there are tremendous improvements in the diagnosis and treatment of aspergillosis, serious fungal diseases still occur and are quite difficult to cure. The death rates are still high, especially among immunocompromised individuals [6]. Development of a timely diagnosis is usually a problem because the current mycological test procedures are unable to control aspergillosis. DNA sequence-related approaches are the most crucial and trusted method in the identification of fungal species compared to the identification of morphological features [7]. Amplification and analysis of the fungal DNA is being used as a fast method to identify the fungi using the ribosomal cistron (rDNA), the internal transcribed spacer (ITS) region [8]. There have been extensive attempts to enhance and compare the process of preparing DNA in fungi by the use of manual and commercial kits. The biggest obstacles are the fracturing of fungal cell walls in the liquid nitrogen or hazardous organic solvents such as phenol [9]. These methods are laborious and costly, especially when commercial kits are employed, and cannot be used in a large sample population. Moreover, the use of solvents that are toxic to human health and the environment is dangerous [10]. Because only a minute amount of crude DNA stimulus can be effectively used to amplify the ITS region in a successful PCR reaction, the present work aimed to design a quick, harmless, and economical method for fungal DNA isolation. For this reason, here the developed DNA extraction protocol was tested for filamentous fungi, particularly *Aspergillus,* in addition to evaluating the accuracy of MAb JF5 for *Aspergillus* antigen detection.

Material and Methods

Fungal strains

Pure cultures of 60 fungal species related to ascomycetes were kindly provided by the Laboratory of Mycological Research (Postgraduate) (LMR). All isolates were refreshed and grown on Potato Dextrose Agar (PDA) (HiMedia, India) that was supplemented with the broad-spectrum antibiotic, Rifampicin, and incubated for around 7 days.

Researchers sterilized the media by autoclaving at 121 °C before use.

Genomic DNA Extraction

For fungal genomic DNA (gDNA), the developed protocol was compared with the standard manual protocol, the CTAB protocol as described by [11]. Using the tested method, biomass was collected from PDA slant cultures by adding approximately.

3 ml of sterile water to facilitate surface scraping by a loop or sterile pipette tips. The washed spores and hyphae fragments were moved into 1.5 ml Eppendorf tubes and centrifuged for 5 min at 14000 rpm. The supernatants were thrown off, and approximately 200 mg of fungal biomass was mixed in a clean Eppendorf tube with 500 µl of 500 mM NaOH (**Figure 1**). The suspensions were mixed at a ratio (1:50) with 150 mM Tris-HCl, vortexed for 30 seconds, and used directly for PCR amplification.

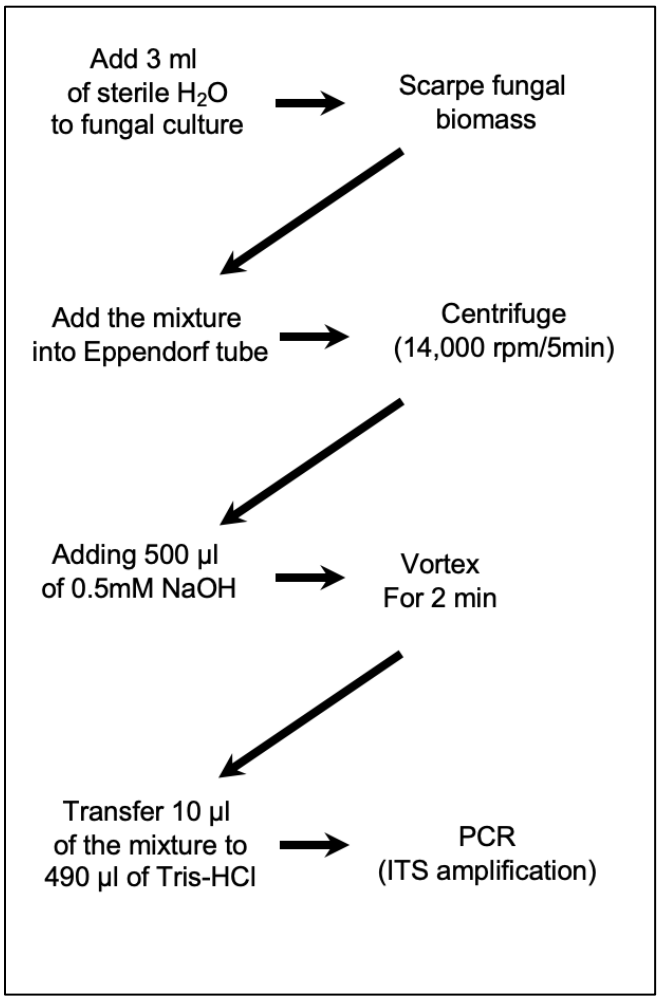

Figure 1. The developed protocol for DNA extraction is diagrammed.

Molecular Fungal Identification

The identification of the fungi was done using the universal ITS primers as described in [12]. The amplification was conducted by adding 1 µl of genomic DNA template, 12.5 µl of 2x Green Master mix, 1 µl of each ITS primer (20 pmol), and 9.5 µl of nuclease-free water to the reaction volume of 25 µll. The PCR amplicons obtained were first confirmed using a 1.5% (w/v) agarose gel. After that, the PCR products were sent to Macrogen (South Korea) to do bidirectional Sanger sequencing. The identification was done on the species level using the obtained sequences and comparing them with the NCBI GenBank database through BLAST.

Immunodetection of *Aspergillus* antigens

For *Aspergillus* antigen detection, the tested fungi were cultivated on PDA slants, and surface washing with water-soluble antigens was performed as indicated elsewhere [13]. Microtiter plates were then coated using 50 □l volumes. Wells were incubated overnight at 4 □C, followed by four phosphate buffer saline (PBS) washes (PBS with tween 20, 0.05 % (v/v)) and distilled H2O, then dried in

a laminar flow hood. The immobilized antigens were blocked with 100 □l blocking solution containing 1% (wt/vol) Bovine Serum Albumin in PBS. The diluted MAb JF5 was conjugated using the Horseradish peroxidase (HRP) kit (Expedeon, UK, #701-0001). MAb JF5 was added to each well after 1 h of incubation. Then, wells were incubated with a TMB (tetramethylbenzidine) as substrate solution for 30 min to visualize bound antibody. Finally, adding 50 □L of 2M H2SO4 as a stop solution and the absorbance value.

We measured the absorbance at 450 nm. We performed all incubations at room temperature in plastic bags. We used only PBS (blank) as the control to determine the antigen threshold in ELISA. Accordingly, we regarded absorbance values above 0.01 as positive for the presence of antigen.

Results

Molecular Identification

The result showed that the tested fungi belonged to 7 fungal genera. Within the tested *Aspergillus* species at the species level, *A. flavus* was the most frequent species, followed by A. niger.

A. terreus, followed by *A. oryzae*, *A. nidulans,* and *A. ruber* (**Tables 1** and **2**).

The optimized protocol in this study provides a great opportunity to obtain fungal DNA within a minute using an alkaline solution with a small amount of fresh or ground fungal biomass. The developed procedure reduced the time-consuming processes involved in fungal DNA extraction as compared to other manual procedures, such as CTAB. This protocol was done using 60 pure fungal isolates related to the Ascomycota phylum. All the tested fungal isolates had a successful percent of ITS amplification, which was 100%. The result of ITS sequencing of the rapid method was verified using the CTAB method, which showed an identical

Table 1. Distribution of tested fungi based on genus.

Fungal genus	Number
Aspergillus	23
Penicillium	9
Parengyodontium	9
Trichoderma	8
Phoma	5
Alternaria	3
Cladosporium	3
Total	**60**

Table 2. Distribution of tested fungi based on species.

Fungal species	No.
Aspergillus flavus	8
Aspergillus niger	6
Aspergillus terreus	4
Aspergillus sp. Mar1	2
Aspergillus oryzae	1
Aspergillus nidulans	1
Aspergillus ruber	1
Penicillium crustosum	4
Penicillium expansum	2
Penicillium echinulatum	1
Penicillium brevicompactum	1
Penicillium griseofulvum	1
Trichoderma asperellum	6
Trichoderma atroviride	1
Trichoderma viride	1
Cladosporium macrocarpum	1
Cladosporium herbarum	1
Cladosporium limoniforme	1
Alternaria alternata	2
Alternaria infectoria	1
Parengyodontium album	9
Phoma herbarum	5
Total	**60**

percentage of the extracted DNA (**Table 3**). The possibility to process a large number of samples, around 60 or 65 samples at a run, is one of the primary benefits of this modified protocol. This amount of extracted DNA and the size were sufficient to be a template for ITS amplification (**Figure 2**). The protocol also removes the hazard of employing toxic chemical solvents in the purification of DNA, like phenol, among others, in addition to minimizing the overall cost in comparison with commercial fungal DNA isolation kits.

Table 3. Efficacy of DNA Extraction Protocol for Fungal Identification.

Feature	Rapid Protocol	CTAB Method
Tested fungal isolates (total)	60	60
Tested fungal genera (total)	7	7
Number of tested species	22	22
PCR amplification rate	100% (60/60)	100% (60/60)
Workflow steps	7	More than 7
Successful identification (%)	100%	100%
Total processing time	~ (5-10) minutes	~ (4 – 5) hours
Toxicity (solvent use)	No	Yes
Multiple tube transfers	No	Yes
Technical skill required	**None**	Professional

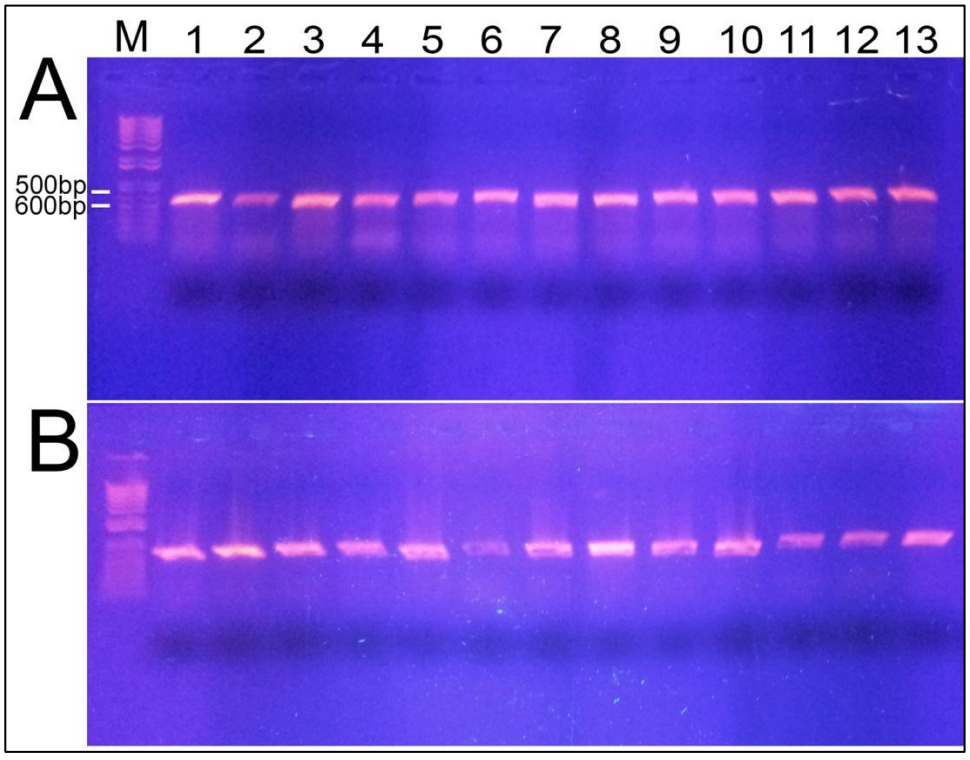

Figure 2. ITS bands developed by agarose gel electrophoresis. The amplified DNA ranged from 500 to 600 bp. A: PCR products of DNA extracted with the rapid method. B: PCR products with the standard manual method by CTAB. M: 1 kb DNA ladder.

ELISA Using JF5

The result of using direct ELISA with MAb JF5 showed a high specificity to *Aspergillus* antigens. The optical density (OD) values of *Aspergillus* isolates were much higher compared to all other fungal species (OD > 1.0) (**Figure 3**). Among the 60 fungal isolates studied using ELISA, 23 (38.3%) isolates were confirmed using ITS sequencing as *Aspergillus* species with a high value of OD. The other isolates that were non-*Aspergillus* represented a variety of non-reactive fungi to MAb JF5, with less than 0.01.

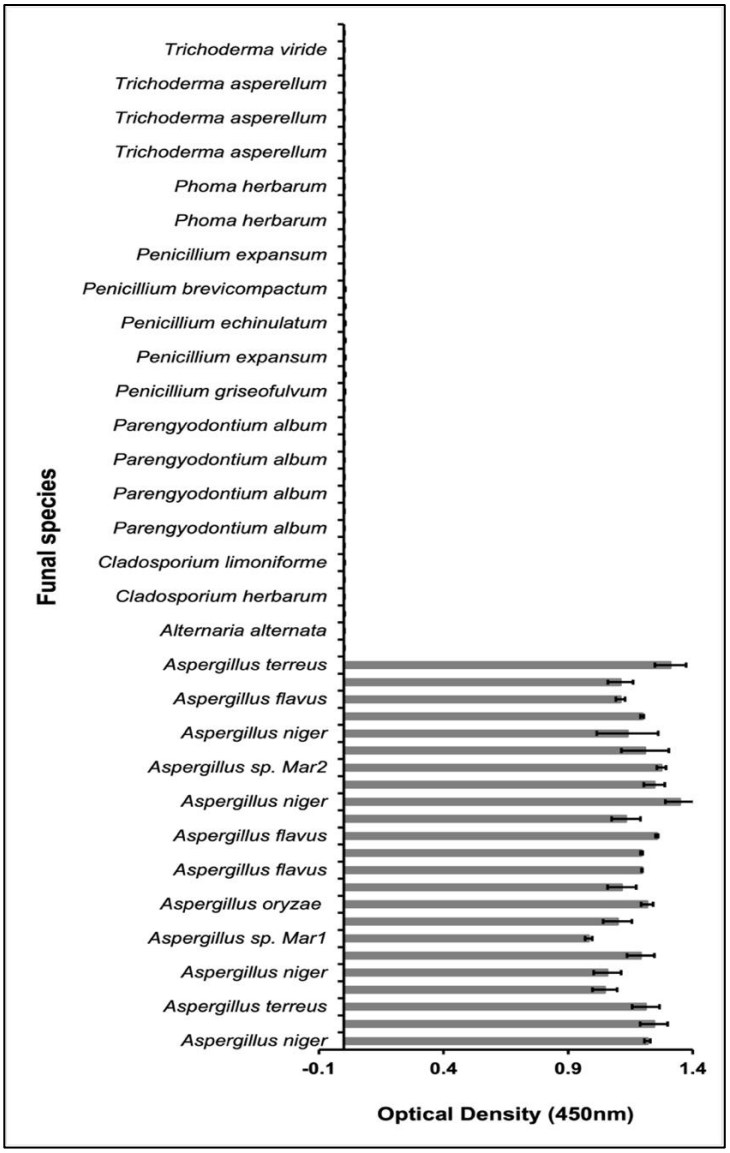

Figure 3. The absorbance values at wavelength 450 nm of direct ELISA using specific monoclonal antibody JF5 to *Aspergillus* antigen.

Discussion

There has been a huge upward trend in the frequency and severity of fungal infection. Diagnosis of fungal infections is an important for successful therapy. It is however, challenged with a number of issues such as reduced clinical mycologists, high cost, long time, and sensitivity and specificity requirements. Moreover, the harmful diagnostic approaches have to serve divergent diagnostic requirements. the necessity to detect a wider profile of fungi in extremely medicalized facilities with widespread immunosuppressive treatment, and the necessity to realize detectives at low costs in resource-restricted areas were opportunistic infections, especially in the immunocompromised patients. The protocol that was developed in the present study to extract fungal genomic DNA completely reduces of the laborious procedures and the costly time-consuming processes. The structure of fungal cell wall consist of glycoproteins, chitin and glucan [14]. It is also found that these components are cross-linked extensively in a group to create a solid structure that link with the cell membrane which includes a distinctive ergosterol and lipid [15]. The cell wall architecture of fungi commonly adds to the challenges of extracting DNA of filamentous fungi and yeasts and undergo prolonged pretreatment procedures [16]. Also, it requires costly reagents such as enzymes and toxic solutions like phenol used in retrieving DNA [17]. In order to remove the use of multi-steps pretreatment to isolate DNA, the protocol that is being developed is to extract DNA from fungal biomass immediately after the treatment of alkaline NaOH solution. The only step that is needed is homogenization of the mixture after a few seconds. We can do our developed protocol in a maximum of two minutes per sample with the addition of alkaline solution which contributed to the reduction of the time value of DNA extraction procedure. It requires working with a large number of samples at a time, which is an important aspect of traditional methods of genomic DNA acquisition because it involves a sterile working area to prevent the contamination of DNA especially in clinical microbiology labs [18]. The procedure that has been developed can be widely applied to diverse types of filamentous fungi that have been isolated either from the environmental samples or clinical samples. It allows more than 65 samples to be processed simultaneously hence enhancing throughput. The ITS region was amplified successfully with universal primers ITS on DNA extracted from fresh mycelium with adequate and high-quality template and allowed the quick identification of fungi [19]. The size of amplified ITS region is located within approximately 450 to 800 bp for the most of fungal species.

The genus *Aspergillus* is associated with a wide range of serious life-threatening infections by causing invasive aspergillosis infections and allergic reactions due to the small size of their spores, followed by aerial sporulation of *Penicillium*, *Alternaria*, and *Cladosporium* [20]. Monoclonal antibodies are a powerful tool in the field of fungal diagnostics because they are highly accurate. They have a high level of specificity in their analysis as they bind to a unique epitope of the target antigen, reducing the possibility of cross-reacting with non-target fungi or host proteins [21]. This sensitivity enables the identification of low-abundance antigens such as *Aspergillus* galactomannan or *Candida* mannan in clinical samples without preceding purification [22]. The MAb JF5 is one of these powerful antibodies specific for *Aspergillus* antigen that can be used to give reproducible and reliable results when incorporated into standardized immunoassays such as ELISA or lateral flow devices [23]. This diagnosis is vital in the initiation of prompt, specific antifungal

treatment, thus enhancing patient outcomes of invasive fungi. The result of the current study validates the specificity and accuracy of JF7 in the detection of *Aspergillus* antigens and can be used to validate the molecular identification. This rapid protocol provides a reliable method with low cost and is safe for fungal DNA extraction. This method has a crucial advantage for screening a large sample of fungi with a high possibility to replace expensive commercial kits.

Conclusion

The developed protocol can be used in the diagnosis of epidemiological work and the diagnosis of filamentous fungi. Such a protocol is fast, dependable, secure, and cheap. MAb JF5 exhibited accuracy in the detection of *Aspergillus* antigens.

Conflict of interest

The authors declare that they have no conflict of interest.

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طريقة بسيط وسريع لاستخلاص الحمض النوويللتشخيص الجزيئي والمناعي لأنواع الفطريات من جنس *Aspergillus*

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جامعة البصرة، كلية العلوم، قسم علوم الحياة

تتمحور هذه الدراسة حول تطوير طريقة سريعة، فعالة، ومنخفضة التكلفة لاستخلاص الحمض النووي الريبوزي (rDNA) من المزارع النقية للفطريات الكيسية (Ascomycetes) ، بهدف تلبية الاحتياجات المتزايدة لطرق التشخيص الجزيئيكبديل للطرق التقليدية في تشخيص الفطريات اعتمادا على المواصفات المظهرية. وقد أثبتت الدراسة أن الطريقة الفطريات الكيسية اذ أوضحت الدراسة إمكانية استخلاص DNA المطور ذات كفاءة استثنائية بنسبة 100% في استخلاص DNA التقليدية لاستخلاص (CTAB) ، وذلك باستخدام عدد كبير من العينات ضمن وقت قياسي عند المقارنة بالطريقة وبالتوازي مع النجاح تلك الطريقة الجزيئية، تم التحقق مناعياً من تشخيص الفطريات التابعة ITS. برايمرات خاصة لتضخيم التي أظهرت دقة عالية في التعرف على (JF5) وباستخدام الأجسام المضادة أحادية النسيلة *Aspergillus* الى الجنس ويتميز هذا الإجراء بكونه بسيطاً، واقتصادياً، وآمناً لعدم اعتماده على (ELISA) عبر تقنية*Aspergillus* مستضدات جنس المذيبات العضوية الخطرة، مع إمكانية معالجة عينات متعددة في وقت واحد.

الكلمات المفتاحية: JF5، *Aspergillus*، جين، التضخيم الجيني، اليزاITS.،