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Original Article

Evaluation of three DNA extraction methods from fungal cultures

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ABSTRACT

Polymerase chain reaction (PCR) based assays have been developed to amplify DNA of fungal pathogens as culture-based detection methods show low sensitivity. In order to perform a sensitive, specific, and reliable PCR based assay, the availability of pure DNA as well as an easy-to-perform DNA extraction protocol is essential. The existing protocols for DNA extraction used for bacteria or viruses show poor release of fungal DNA. In this study, we evaluated three different methods of DNA extraction and compared their efficacy in the extraction of DNA from filamentous fungi, yeasts, and dermatophytes commonly isolated in our laboratory. It was found that the Fungi/Yeast Genomic DNA Isolation Kit (Norgen Biotek Corp, Ontario, Canada) demonstrated satisfactory extraction of DNA from all the fungi analyzed as compared to that of the Qiamp DNA extraction kit (Qiagen GmbH, Dusseldorf, Germany) or the Phenol Chloroform Isoamyl alcohol extraction method which failed to extract amplifiable DNA from many of the fungal species. Thus, we recommend the use of Fungi/Yeast Genomic DNA Isolation Kit (Norgen) with modifications for the extraction of DNA from fungal cultures.

Introduction

Polymerase chain reaction (PCR) based assays have been developed to amplify DNA of fungal pathogens as culture-based isolation (detection) methods show low sensitivity and poor specificity.[1–3] The existing protocols for DNA extraction used for bacteria or viruses show poor release of fungal DNA. This is owing to the fact that fungi possess a rigid, laminated, and complex cell wall that protects and provides firmness to its cell structures. The cell wall of filamentous fungi consists of an inner microcrystalline sleeve of chitin [poly-β-(1→4)-N-acetylglucosamine], while in the case of yeast cells chitin is concentrated as disks in their cell wall. Thus the presence of a complex fungal cell wall makes it resistant to lysis by the commonly used methods and impairs extraction of the fungal DNA.[4] Many of these methods recommend grinding with mortar and pestle or freeze drying with liquid nitrogen to assist the breaking up of the mycelia. This entails significant increase in the time frame as well as requirement of liquid nitrogen, when dealing with a large number of specimens. Invasive fungal infection is an important cause of morbidity

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and mortality particularly in the immunocompromised patient requiring rapid diagnosis and therapy. The availability of a reliable fungal DNA extraction method will reduce the turnaround time considerably.[5] Studies have evaluated the manual and commercial methods of fungal DNA extraction such as boiling, glass beads for bead beating, sonication, and washing with alkaline heat lysis and methods such as QIAmp tissue, GeneReleaser DNA, and Gentra Puregene tissue kit. It was found that the heat alkali enzymatic digestion protocol, and the QiAmp tissue kit performed the best.[5] Proteinase K and lyticase are enzymes that catalyze fungal cell lysis by β-1,3-glucanase and a specific alkaline protease activity. These properties have been exploited in in vitro production of protoplasts or spheroplasts during DNA extraction from fungi.[6] In the present study, we evaluated three different methods of DNA extraction namely (a) the phenol chloroform isoamyl alcohol (PCI) method, (b) Spin-column method using a Qiagen DNA extraction kit and (c) Spin column method using Norgen DNA extraction kit, both employing prior disruption of cell wall by bead beating and sonication and pretreatment with lyticase and proteinase K enzymes (details of the procedures mentioned in the results section). The efficacy in the extraction of DNA from various fungal species, was checked by amplification with primers targeting the Intergenic Transcribed Spacer (ITS) region of the ribosomal DNA.[7]

Table 1 – List of standard and clinical strains of Fungi used for the extraction of DNA along with the approximate amplicon size for each fungus.

ATCC strain number/isolate	Organism	Approximate base pair (bp) size of band on gel electrophoresis
ATCC 90028	*Candida albicans*	~215
ATCC 750	*Candida tropicalis*	~215
ATCC 6258	*Candida krusie*	~215
ATCC 22019	*Candida parapsilosis*	~215
ATCC 6258	*Candida guillermondii*	~215
ATCC 208821	*Cryptococcus neoformans*	~200
ATCC 293	*Aspergillus fumigatus*	~300
ATCC 204304	*Aspergillus flavus*	~300
ATCC 9508	*Aspergillus niger*	~300
Clinical isolate	*Aspergillus terreus*	~300
Clinical isolate	*Aspergillus nidulans*	~300
Clinical isolate	*Aspergillus glaucus*	~300
Clinical isolate	*Mucor* spp.	~300
Clinical isolate	*Rhizopus* spp.	~300
Clinical isolate	*Penicillium* spp.	~280
Clinical isolate	*Fusarium* spp.	~225
Clinical isolate	*Trichophyton rubrum*	~220
Clinical isolate	*Microsporum gypseum*	~220
Clinical isolate	*Epidermophyton mentagrophytes*	~220
Clinical isolate	*Trichosporon asahii*	~290

Results

Three methods for the extraction of fungal DNA from standard and clinical strains were tried out, which included the PCI method and two Spin column methods: DNA extraction kit (Qiamp mini kit, Qiagen GmbH, Germany), and Fungi/Yeast Genomic DNA Isolation Kit (Norgen Biotek Corp, Ontario, Canada). Fungal isolates included in the study were *Candida albicans* (ATCC 90028), *Cryptococcus neoformans* (ATCC 208821), and *Aspergillus fumigatus* (ATCC 293) and archived laboratory confirmed clinical isolates of *Rhizopus* spp., *Fusarium* spp., *Microsporum gypseum*, *Epidermophyton floccosum*, *Penicillium* spp. and *Trichosporon asahii* (Table 1). The DNA extraction procedures were carried out in a Class 2 Biosafety cabinet in a dedicated room with suitable biosafety precautions and appropriate biowaste disposal methods.

PCI method of DNA extraction: Formerly used in our laboratory for extraction of DNA from *Mycobacterium tuberculosis*.

Fungal mycelia or 2–3 colonies of yeast were taken in a 1.5 mL micro-centrifuge tube to which 1 mL of Phosphate buffered saline was added. The tube was vortexed using a vortexer (Remi, Mumbai, India) and then was centrifuged and 250 μL of lysis buffer (10% SDS, 0.5 M EDTA, 17.5% NaCl and 10 M NaOH) was added, incubated at 95 °C for 20 min followed by addition of 500 μL of PCI in the ratio of 25:24:1. The mixture was vortexed and then centrifuged in a refrigerated micro-centrifuge (Eppendorf, Hamburg, Germany) at 10,000 rpm for 15 min at 4 °C. A mixture of chloroform–isoamyl alcohol (480 μL + 20 μL) was added to the supernatant, centrifuged at 10,000 rpm for 15 min at 4 °C. Sodium acetate (0.3 M) and isopropanol at 0.1 and 0.7 volumes respectively were added to the supernatant and incubated at room temperature for 30 min, then centrifuged at 12,000 rpm for 15 min. The sediment containing DNA was washed twice with chilled ethanol (4 °C/200 μL) at 6000 rpm for 2 min and dried at 68 °C for 5 min. The DNA was re-suspended in 30 μL of Tris–EDTA buffer and stored at −20 °C for further processing.

*DNA extraction by Qiamp Mini Kit (Qiagen) with modifications**

Extraction was done as per the manufacturer's instructions with minor modifications. Briefly, fungal mycelia/yeast were pelleted in phosphate buffer saline by centrifugation for 10 min at 7500 rpm and resuspended in 180 μL of 2 mM EDTA solution, incubated for 30 min at 37 °C. Twenty microliters of proteinase K (Qiagen, USA) and 200 μL of lysis buffer were added and the mixture was vortexed and incubated at 56 °C for 30 min followed by 95 °C for 15 min to deactivate the proteinase K. Following a brief spin down, 200 μL of lysis buffer was added, and the mixture was transferred to a *Bead Tube and vortexed on a vortexer* (Remi, Mumbai, India) *for 10 min at maximum speed followed by incubation at 90 °C for 2 h with intermittent vortexing. The bead tube was sonicated for 20 min in an ultrasonication water bath (Elmasonic, Germany)**.[6] The mixture was transferred to the QIAamp Mini spin column after addition of 200 μL of chilled (4 °C) ethanol. The tube was washed twice with 500 μL wash buffer by centrifuging each time at 8000 rpm for 1 min. The DNA was eluted after addition of 200 μL elution buffer and by centrifuging at 8000 rpm for 1 min and stored at −20 °C till further use.

*Fungi/Yeast Genomic DNA Isolation Kit (Norgen) with modifications**

The extraction procedure was followed as per the manufacturer's instructions with some modifications. 5 mL of

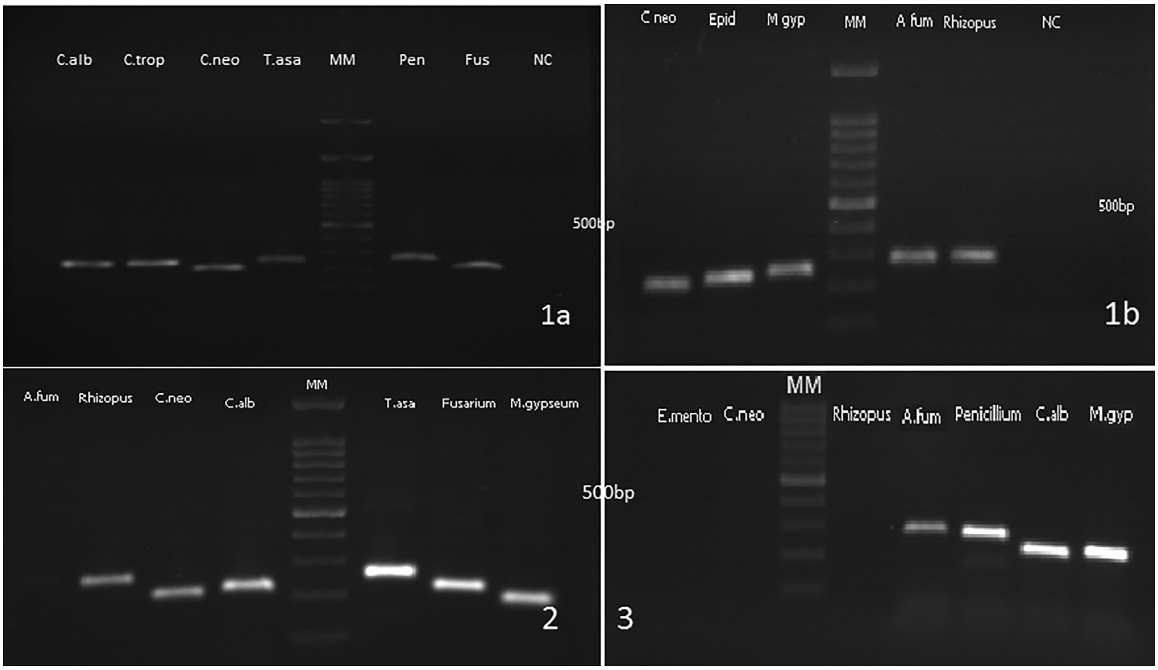

Fig. 1 – Gel electrophoresis of amplicons generated from DNA extracted by: (Panel 1a and 1b) Fungi/Yeast Genomic DNA Isolation Kit (Norgen). (Panel 2) Qiagen DNA mini kit (QIAamp, Qiagen). (Panel 3) Phenol chloroform iso-amyl alcohol method. C.alb – *Candida albicans*, C.trop – *Candida tropicalis*, C.neo – *Cryptococcus neoformans*; T.asa – *Trichosporon asahii*, Pen – *Penicillium* spp., Fus – *Fusarium* spp., Epid – *Epidermophyton mentagrophytes*, M.gyp – *Microsporum gypseum*, A.fum – *Aspergillus fumigatus*, Rhizopus – *Rhizopus* spp., Fusariium – *Fusarium* spp., M.gyp – *Microsporum gypseum*, E.mento – *Epidermophyton mentagrophytes*, Penicillium – *Penicillium* spp., NC – negative control, MM – 100 bp molecular marker (500 bp is marked).

collection solution was poured on the culture plate, and the fungal mycelium was pelleted in a microcentrifuge tube by centrifugation at 14,000 rpm for 1 min. Two hundred fifty microlitres of Resuspension Solution A, *200 units of lyticase* enzyme* (Sigma Aldrich, USA)[8] and 150 μL of proteinase K were added to the cell pellet and incubated for 45 min at 37 °C. Five hundred microlitres of lysis buffer was added, and the mixture was vortexed in a bead tube on a vortexer for 10 min at maximum speed followed by incubation at 90 °C for 2 h with intermittent vortexing. *The bead tube was sonicated for 20 min in an ultrasonicator water bath**.[6] After centrifugation for 2 min at 14,000 rpm, the supernatant was transferred to a fresh microcentrifuge tube with equal volume of ethanol and vortexed. The lysate was placed in the Spin column and centrifuged at 10,000 rpm for 1 min followed by two washes with 500 μL of wash buffer. The DNA was eluted in 100 μL of elution buffer (composition not mentioned in insert) by spinning at 10,000 rpm for 2 min and was stored at −20 °C till further processing.

The extracted DNA was run on a 1% gel to assess the quality (data not shown). PCR was carried out to amplify the fungal DNA using forward primer ITS5 (GGAAGTAAAAGTCGTAA-CAAGG) and reverse primer ITS2 (GCTGCGTTCTTCATC-GATGC) targeting the ITS region of 18S rRNA.[7] Briefly, the cycling parameters included the following initial denaturation 10 min at 95 °C followed by 40 cycles of denaturation for 30 s at 95 °C, annealing for 30 s at 50 °C, final extension for 07 min at 72 °C. The amplified products were electrophoresed in a 2% agarose gel and visualized on an Ultra Violet transilluminator (Uvitec, Cambridge, UK) using a 100 bp molecular marker (Thermofisher Scientific, USA) for marking of approximate base pair sizes (Table 1) (* – modification mentioned in italics).

The amplicons generated by various fungi from the DNA extracted by Fungi/Yeast Genomic DNA Isolation Kit (Norgen) following amplification with the above-mentioned primers are shown in Fig. 1: panels 1a and 1b. Amplicons after extraction by Qiamp DNA extraction mini kit and PCI method are shown in Fig. 1: panel 2 and panel 3, respectively. Template DNA extracted by the Fungi/Yeast Genomic DNA Isolation Kit (Norgen) with modification produced amplicons in the PCR carried out on all the fungal strains studied. QIAamp DNA extraction kit with modification failed to extract amplifiable DNA from *A. fumigatus* and *Penicillium* spp. as seen by the absence of bands in post amplification gel electrophoresis for these two fungi (Fig. 1 panel 2). Phenol chloroform method failed to extract amplifiable DNA resulting in no amplicons from Zygomycetes (*Rhizopus* spp. and *Mucor* spp.), *C. neoformans*, and *Epidermophyton mentagrophytes* as depicted in Fig. 1 panel 3.

Discussion

Generally, obtaining DNA from fungi is more difficult than from bacteria or from mammalian cells as the fungal cell wall is tough to break using the conventional extraction methods employed for bacteria or viruses. This is attributed to fungi possessing a thick and complex cell wall resulting in poor release of DNA. Poor efficiency of DNA extraction, low numbers of fungal cells in clinical material may be the reasons for obtaining false negative results from clinical specimen.[6] Over the last two decades, many molecular methods have been extensively tested for the detection of fungal DNA in blood and in other clinical samples.[5] Therefore, in addition to using specific primers and probes, suitable for the detection of low level of fungal DNA, it is extremely important to use the most efficient method of DNA extraction. Additional procedures leading to disruption of the fungal cell wall (e.g., by mechanical, enzymatic and/or chemical methods) are required.[6] Experts from the United Kingdom Fungal PCR Consensus Study Group and the European Aspergillus PCR Initiative (EAPCRI) working group of the International Society of Human and Animal Mycoses[9] while recommending the use of PCR in the diagnosis of mycoses, agree that extraction procedure is the main factor limiting the efficiency of PCR.

Many protocols for the extraction of fungal DNA from clinical specimens employing different commercial kits have been described in the medical literature.[6,10]

Griffiths et al. have reported extraction with a Qiagen kit utilizing bead beating technique along with a lysis buffer where they found the yield of DNA better than other methods.[6] Van Burik et al. evaluated the use of glass beads with vortexing for extended periods and found it better for extraction of DNA from filamentous fungi.[10]

Currently, the available kits are intended for isolation of human DNA from clinical samples require additional pretreatment for fungal cell disruption.

Here, we have described methods that can be employed in a routine laboratory and that do not involve either freezing using liquid nitrogen or grinding. In our study, the QiAmp kit method failed to extract amplifiable DNA from some of the fungi (*A. fumigatus*). While the phenol chloroform extraction method is cheaper, it failed to extract amplifiable DNA from some of the filamentous fungi and yeast (*Rhizopus* spp. and *C. neoformans*). Moreover, phenol being a toxic chemical is nowadays avoided in routine practice. The best amplification was obtained after extraction with the Norgen kit with addition of a proteinase K digestion step over and above lyticase digestion, followed by bead beating and sonication. This method extracted amplifiable DNA from all the fungal strains studied. The time taken by every kit method was similar to each other. Costing of the Qiagen and the Norgen kits were similar. In this study, we found that using the Norgen Fungi/Yeast Genomic DNA Isolation Kit with modifications for the extraction of DNA from fungal cultures yielded better results for isolation of fungal DNA. Overall, the Norgen kit method was the most efficient method in the extraction of DNA from fungal cultures compared to the other methods considering time, cost, technical expertise, and instrumentation. Further evaluation on clinical samples is required in order to check the efficacy of the extraction.

Conflicts of interest

The authors have none to declare.

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