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Journal of Microbiological Methods 56 (2004) 431–434
Journal of Microbiological Methods
www.elsevier.com/locate/jmicmeth

Note

A rapid DNA extraction method for PCR identification of fungal indoor air contaminants

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Received 7 November 2003; received in revised form 18 November 2003; accepted 18 November 2003

Abstract

Following air sampling fungal DNA needs to be extracted and purified to a state suitable for laboratory use. Our laboratory has developed a simple method of extraction and purification of fungal DNA appropriate for enzymatic manipulation and Polymerase Chain Reaction (PCR) applications. The methodology described is both rapid and cost effective for use with multiple fungal organisms.
Published by Elsevier B.V.

Keywords: PCR inhibitors; DNA extraction; *Stachybotrys chartarum*

The Polymerase Chain Reaction (PCR) has become a preferred method for the identification of biological indoor air contaminants. To this end, techniques have been developed to sample and purify filamentous fungal DNA from air, water, and building surfaces. The primary goal of these techniques is to improve the sensitivity of detection as well as limit the time it takes to prepare the samples for PCR analysis. Recent work has enabled laboratories to extract and purify PCR quality DNA templates in times ranging from 2 h to 90 min (Haugland et al., 2002; Roe et al., 2001). One common feature of the extraction and purification of fungal DNA is the use of specialized kits and reagents to remove PCR inhibitors. Our goal was to develop a rapid method of DNA extraction and purification from *Stachybotrys chartarum* from air samples suitable for PCR or quantitative PCR (qPCR) utilizing common laboratory reagents, while at the same time maintaining high sensitivity and accuracy in the PCR reaction. Our decision to use *S. chartarum* was based on its implication in sick building syndrome (Mahmoudi and Gershwin, 2000; Kuhn and Ghannoum, 2003).

Liquid spore suspensions of *S. chartarum* were prepared according to the method of Crow et al. (1994). Basically, spores were harvested from plates with 3 ml of 0.01 M phosphate buffer with 0.05% (v/v) Tween 20 (Sigma Chemical, St. Louis, MO, USA) by gently agitating the plate surface with a bent glass rod. The supernatant from numerous plates was combined and the spore suspension centrifuged at 12,000 × *g* for 5 min. The supernatant was decanted leaving the spore

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0167-7012/$ - see front matter. Published by Elsevier B.V.
doi:10.1016/j.mimet.2003.11.015

pellet intact. The pellet was washed three times with 10 ml of phosphate buffer and stored at 4 °C until needed. Total spore counts were enumerated by direct microscopic counting on a hemacytometer as described by Roe et al. (2001).

Extraction of genomic DNA from the spores was accomplished using a bead milling method. Previous work has shown that disrupting *S. chartarum* spores suspended in water is just as effective for harvesting DNA as using reagents that facilitate microbial cell lysis (Haugland et al., 2002). The bead milling method involves adding 0.25 g of acid-washed glass beads (212–300 μm) to a 2.0 ml conical screw cap tube. To the beads, 200 μl of spore suspension (approximately 3.2×10^7 spores/ml) was added, and the tube was shaken in a mini bead beater (Biospec Products, Bartlesville, OK) for 50 s at the maximal rate. The mixture was then cooled on ice for 1 min and subjected to another 50 s in the bead beater. The bead spore mixture was briefly spun down at 5000 rpm in a bench top centrifuge, and the supernatant removed from the beads using a 200 μl pipette tip. This "crude" DNA extract was then used in the initial PCR trials.

All PCR reactions included 1 μM each of forward primer IT51 (5′-GAGGAAGTAAAAGTCGTAACAAGGT-3′) and reverse primer IT43 (5′-CTTTTCCTCCGCTTATTGATATGC-3′; Haugland et al., 1999). This primer pair amplifies ribosomal DNA sequences including a portion of the small subunit ribosomal DNA, the internal transcribed spacer regions 1 and 2, the entire 5.8S ribosomal DNA, as well as a portion of the large subunit ribosomal DNA, generating a PCR product approximately 600 bp in length. Each reaction contained: 0.2 mM each dNTP, 1.5 mM $MgCl_2$, 1.5 U Taq DNA polymerase, Buffer (50 mM KCl, 10 mM Tris–HCl, pH 9.0 at 25 °C, 0.1% Triton X-100), and variable template concentrations. PCR was performed for 27 cycles of 96 °C for 30 s; 50 °C for 15 s; and 68 °C for 4 min. PCR products were separated by electrophoresis in 2% low melting point agarose and were visualized by ethidium bromide staining. To confirm that the proper sequence was being amplified, initial PCR products were sequenced using an ABI 3100 Genetic Analyzer, and the output sequences were compared to *S. chartarum* strain ATCC 9182 accession number AF081468.

The presence of PCR inhibitors in *S. chartarum* samples have been reported (Cruz-Perez et al., 2001). PCR inhibitors are commonly found in environmental samples and may include pollen, humic material, and biological fluids. Initial PCR results with the "crude" *S. chartarum* DNA extract revealed the presence of PCR inhibitors (Fig. 1). At concentrations of spores above 10^4, the reaction produced a negative result. However, sensitivity was very good with positive results seen at concentrations as low as 10^2 spores in the reaction. Incorporation of an internal positive control (purified *S. chartarum* DNA) in the PCR reaction demonstrated the presence of inhibitors in these samples. Furthermore, dilution of the negative reactions produced positive PCR results (data not shown). The same experiments were carried out with *Aspergillus niger* and *Cladosporium sphaerospermum* showing decreased polymerase activity at higher concentrations of spores, indicating the occurrence of PCR inhibitors in samples of indoor fungal contaminants.

In order to identify indoor air contaminants by PCR, it is essential to remove all PCR inhibitors, or negative amplification reactions can result. In order to extend the purification of the extracted fungal DNA, a phenol:$CHCl_3$ extraction was utilized followed by ethanol concentration. Standard methodology was performed following Ausubel et al. (1994). A 1:1 phenol:$CHCl_3$ mixture was added to an equal volume of the "crude" DNA extract. After vigorous vortexing for 1 min, the mixture was spun at 16,000 rpm in a microcentrifuge and the aqueous layer was removed. Following a similar treatment with 24:1 $CHCl_3$:i-

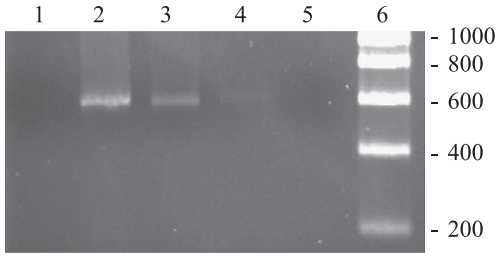

Fig. 1. Results showing the 600 bp amplification product from *S. chartarum* using unpurified extracted ribosomal DNA. Lane 1 contains 10^5 spores; lane 2 contains 10^4 spores; lane three contains 10^3 spores; lane 4 contains 10^2 spores; and lane 5 contains 10 spores. Lane 6 shows the size marker with sizes (in bp) on the right.

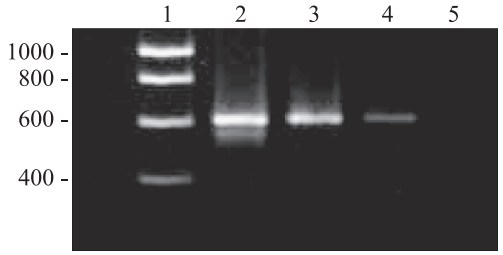

Fig. 2. Results showing the 600 bp amplification product from *S. chartarum* ribosomal DNA following extraction and purification via Phenol:$CHCl_3$ and ethanol concentration. Lane 1 shows the size marker with sizes (in bp) on the left. Lane 2 contains 10^5 spores; lane 3 contains 10^4 spores; lane 4 contains 10^3 spores; and lane 5 contains 10^2 spores.

soamyl alcohol, the extracted DNA was ethanol precipitated and resuspended in dH_2O.

PCR on the purified DNA was completed exactly as previously described. Results were positive (Fig. 2) showing amplification products at concentrations as high as 10^5 spores per reaction. Of further note was a slight loss in sensitivity in the reaction with dependable amplification down to 10^3 spores per reaction. Various reactions did produce faint amplification at 10^2 spores per reaction.

Initial trials showed that phenol:$CHCl_3$ removed the PCR inhibitors that were present in the spore preparations. To test the efficacy of the procedure in a ''real world'' environment, 3400 l ambient air samples were taken (as described below); dust was extracted from the filters and then spiked with 10^7 *S. chartarum* spores.

Briefly, the procedure involved taking air samples using an Anderson Instruments air-sampling pump designed for a flow rate of 28.3 l/min. The pump was attached to a filter holder containing a 37 mm 0.8 μm mixed cellulose ester filter. The pump and filter were then placed outside a residential home 30 ft from the exterior walls. Placement outside allowed for the maximal collection of particulate matter including pollen and other PCR inhibitors. The method required the sampler to be operated at a flow rate of 28.3 l/min for a 2-h period totaling 3400 l. Following sampling, the filters were removed from their respective holders, and the dust was extracted into 1.5 ml 0.01 M phosphate buffered Tween. The dust was then spiked with known quantities of *S. chartarum* spores and subjected to the previously described DNA extraction and purification regimen. To increase the sensitivity of the PCR detection, the number of cycles of PCR was increased from 27 to 30.

The results of the dust-spiked amplification were favorable (Fig. 3). The presence of environmental dust in the samples did not affect the purification and subsequent amplification of the fungal DNA. Phenol:chloroform extraction removed the PCR inhibitors, while, at the same time, increasing the number of PCR cycles allowed for the detection of spores at the lowest concentrations tested. Dust samples that were not spiked with spores failed to generate an amplification product, indicating that there was no background amplification of *S. chartarum* (data not shown).

The extraction and purification of filamentous fungal DNA using the above methodology can be routinely completed in 60 min with the complete removal of PCR inhibitors. This simplified method retains high sensitivity with the capability of detecting very low concentrations of indoor air contaminants. Research has shown that, for all extraction methods tested, nested PCR was more sensitive than either single-step PCR or PCR followed by Southern blotting (Williams et al., 2001). The procedures described here produce very pure DNA lending themselves well to the various PCR systems as well as to newer quantitative PCR (qPCR) methods, which obtain higher sensitivity by using fluorescence detection, and downstream sequencing reactions. Our laboratory currently uses this extraction and purification method

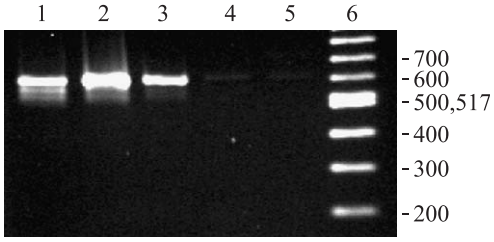

Fig. 3. Results showing the 600 bp amplification product from *S. chartarum* ribosomal DNA with environmental dust following extraction and purification via Phenol:$CHCl_3$ and ethanol concentration. Lane 1 contains 10^5 spores; lane 2 contains 10^4 spores; lane 3 contains 10^3 spores; lane 4 contains 10^2 spores; and lane 5 contains 10 spores. Lane 6 shows the size marker with sizes (in bp) on the right. Increased cycles of PCR enhance amplification at the lower concentrations of spores.

to prepare suitable DNA for qPCR reactions as well as for sequencing fungal genes (data not shown).

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