METHODS REPORT

Quantitative Real-Time Polymerase Chain Reaction (qPCR) of Filamentous Fungi in Carpet

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Contents

Abstract

We developed a protocol for the rapid identification and quantitation of fungi by quantitative real-time polymerase chain reaction (qPCR) in carpet. The fungi used in this study are field isolates of *Alternaria alternata, Aspergillus versicolor, Cladosporium cladosporoides,* and *Stachybotrys chartarum*. The modified spore extraction method provided superior quality, high-molecular-weight genomic DNA as assayed using SYBR Green I qPCR. The species-specific target sequences were selected from the highly conserved nuclear ribosomal RNA (rRNA) region of fungi. Primer sets produced consistent species-specific PCR products, and we confirmed the target by melt-curve analysis. The qPCR assay had a range of detection of 40 to 25,000 spores per reaction and required less than 60 minutes to run, and the results were reproducible (average $r = 0.95$). The use of this method for genomic DNA isolation from fungi spores coupled with the qPCR using the primer sets we designed will enable quicker identification of disease-causing fungi in the built environment.

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Introduction

Carpet, especially when dirty, can create a suitable environment for fungal growth, especially under damp conditions.[1] Exposure to mold (fungi) in the indoor environment represents a major conduit of respiratory disease in humans, with asthma and allergy being the two most recognized illnesses.[2-4] Because inhalation of fungi has been shown to increase nonspecific bronchial reactivity in asthmatics and has been implicated in the exacerbation of allergy and chronic infection,[5,6] quickly identifying fungi at the species level is crucial for implementing appropriate disease treatment. However, fungi infestation is frequently composed of a mixed population of genera that tends to interfere with the timely identification of the disease-producing fungus.

Although considerable knowledge about fungi has been amassed, they are not rapidly identified by culture morphology because of their prolonged growth requirements and fastidious nature.[7] These factors undoubtedly lead to an understatement of the actual diversity of the indoor fungal community. The need for rapid identification of the species of fungus has led to the development of various molecular technologies; the quantitative real-time polymerase chain reaction (qPCR) lies at the forefront of these technologies.

qPCR can both detect and quantify organisms based on a specific sequence of DNA in the sample. As PCR product accumulates in the reaction, it is quantified in real time. The most common method to detect PCR product is through the use of the fluorescent dye SYBR Green I. SYBR Green I dye intercalates in the double-stranded DNA, amplified by the PCR; quantity is directly proportional to the amount of fluorescence.

A favorite target for designing a qPCR assay is the repetitive and highly conserved nuclear ribosomal RNA (rRNA) region of fungi.[8-13] Although highly conserved, the variation in the intron spacer segments of the rRNA region is sufficient to allow for specific and rapid PCR-based speciation. Although qPCR is a particularly promising molecular tool because of its simplicity, specificity, and sensitivity, qPCR does have some important limitations. These limitations include the availability of sequence data, adequate preparation of inhibitor-free target DNA, and judicious primer design. Users of qPCR must give considerable attention to the DNA purification process, especially when microorganisms are difficult to lyse. Inefficient lysis leads to substandard nucleic acid purification and insufficient removal of compounds inhibitory to PCR.[14,15]

Spores of fungi are favored for laboratory analyses because they are relatively large and easily isolated, have distinguishable characteristics, are countable, and have long viability. However, use of spores for molecular studies like PCR is challenging. Spores are highly resistant to lysis, and several studies have been devoted to the development of procedures to efficiently extract genomic DNA from fungi and bacteria spores.[16-24] Although the methods for genomic DNA isolation are variable, all but one of these previous studies[17] utilized serial dilution of a singular genomic DNA preparation for PCR, and none demonstrated the nature of the genomic DNA that was isolated by agarose gel electrophoresis.

Clearly, a distinctive approach for the rapid isolation of quality high molecular weight genomic DNA from fungi spores is not at all well defined. Although commercial kits are available for DNA isolation from many different types of organisms and cells, none will isolate DNA from fungi spores very well.

This report describes the experimental procedures we used to overcome the unique difficulties in isolating genomic DNA from fungi spores. We combined several techniques to isolate high-molecular-weight genomic DNA from the inoculum. We then used the purified DNA successfully in qPCR, which correctly identified and quantitated each of the fungi isolated from carpet by spore number.

Methods and Materials

Reagents and Kits

We used a Qiagen Plant kit (Valencia, California) for genomic DNA isolation. The kit and components were stored at room temperature until they were needed for our work. We stored the SYBR Green Supermix (BioRad, Hercules, California) used for qPCR at –20°C until it was needed.

Fungi

The test organisms were field isolates of *Alternaria alternata, Aspergillus versicolor, Cladosporium cladosporoides,* and *Stachybotrys chartarum* filamentous fungi. We cultured all test organisms for 14 to 21 days at room temperature under incandescent light on 2 percent malt extract agar (MEA). We gently rolled a swab wetted with sterile water across the surface of growth to collect the spores. We then eluted the material collected on the swab into sterile water and verified spores using light microscopy. Finally, we adjusted spores using hemacytometer to 10^7 spores/mL in sterile water and stored them at 4°C until needed.

Carpet Inoculation and Extraction

We punched circles approximately 5 cm in diameter from commercial-grade nylon carpet and sterilized them by autoclaving. Then we rolled autoclaved, sieved vacuum cleaner dust onto sterile carpet at 0.09 mg/cm^2. The carpet samples were placed back into an autoclave pouch and stored at room temperature until ready for use. We mixed each fungi suspension well before pipeting 50 µL on triplicate carpet punches. Punches were inoculated with 40, 200, 1,000, 5,000, or 25,000 spores. We prepared triplicate sets of controls for each inoculum level.

We extracted spores by shaking a wrist-action shaker in phosphate-buffered saline. We plated the controls on 2 percent MEA and incubated them until colony-forming units (CFUs) could be determined. The CFUs were later used as indicators of spore numbers for extraction of DNA and qPCR tests.

DNA Preparation

We subjected extracted spores to glass bead milling for 5 minutes in the lysis buffer supplied with the Qiagen kit. Cell debris and glass beads were removed from the milled lysate by passing the lysate through Whatman cellulose filter paper (Whatman Inc., Florham Park, New Jersey) at low-speed centrifugation (Eppendorf Centrifuge 5415D, Westbury, New York). We then subjected the cleared lysate to further DNA purification following the manufacturer's suggested protocol. All extractions were eluted in sterile distilled water, concentrated (Centrivap DNA Systems, Labconco, Kansas City, Missouri), and stored at –20°C until needed.

qPCR Primers

We designed all rRNA primers from the following National Center for Biotechnology Information loci: AF314580 (*Alternaria alternata*), L76745 (*Aspergillus versicolor*), AJ244241 (*Cladosporium cladosporoides*), and AM180510 (*Stachybotrys chartarum*). Primers were validated using two software packages, Oligo 3.0 (free Web software) and Molecular Beacon (Premiere Biosoft International, Palo Alto, California). The primers were synthesized by Qiagen Operon (Germantown, Maryland), suspended in sterile water, and stored at –20°C until needed.

PCR Conditions

We evaluated several thermalcycling parameters for optimal performance at amplifying DNA from these fungi spores. Generally, the most effective procedure utilized 5 µL of the purified DNA preparation added to a 20-µL master mixture (made immediately before use) containing 12.5 µL PCR SYBR Green I Supermix (BioRad), 0.25 µM of each primer, and 6.25 µL nuclease-free water (Promega, Madison, Wisconsin). The iCycler (BioRad, Hercules, California) parameters were the following: 3 minutes at 95°C (to denature double-stranded DNA) followed by 40 cycles each of 10 seconds at 94°C, 10 seconds at 57°C, and 10 seconds at 72°C, and a final extension cycle for 5 minutes at 72°C. In addition, we performed a melt curve to confirm that we had generated only the desired amplicon.

Results

Inoculation, elution, culture, and enumeration of the fungi spores extracted from the carpet samples proved to be very consistent (Table 1). This consistency across these variables was important because it provided a basis for determining how many spores were actually extracted for the DNA assays. In this study, the standard deviations were lower in the higher inoculums (2.30–4.40) and higher in the lower

inoculum (1.60). This was somewhat expected based on previous experimentation in which loss of a single spore in lower inoculations resulted in larger error (data not shown).

Although the Qiagen Plant kit is designed to extract DNA directly from plant material, we could not extract the DNA from the fungi spores by following the manufacturer's instructions alone (data not shown). Instead, we had to modify the manufacturer's protocol by milling the spores in lysis solution before DNA extraction. In addition, the milled lysate required centrifugation through filter paper at low speed to remove cell debris and beads.

The DNA that we purified from all four genera after these modification steps was determined by agarose gel electrophoresis to be well-defined, high-molecular-weight genomic DNA (Figure 1). The milled spores photograph (right panel of Figure 1) shows long DNA smears with high-intensity DNA bands at the top of the gel. The majority of the purified genomic DNA corresponds to between 10 and 20 kilobases, as compared with the DNA ladder (lane 1). Conversely, the unmilled spores photograph (left panel of Figure 1) demonstrates the complete absence of detectable genomic DNA in the preparation.

The thermalcycling parameters and primer sets used for this study resulted in qPCR specificity (+) for each of the fungi (Table 2). Each qPCR reaction contained DNA from all four fungi but primers specific for only one. When we ran qPCR, detection of a single amplified product of the same kilobase molecular weight as that of the positive control of the single organism and specific primer set indicated specificity (+). A negative control reaction that contained specific primers but no DNA was included in every qPCR. In each case, the positive control

Table 1. Organism, initial inoculate, and recovered mean inoculum colony-forming units per carpet piece

Organism	Hemacytometer-Verified Inoculum (Log_{10})				
	1.60	2.30	3.00	3.70	4.40
	Actual Recovery (Log_{10}, ± *sd*, n=3)				
Alt. alternata	1.62 ± *0.18*	2.43 ± *0.02*	3.08 ± *0.03*	3.70 ± *0.07*	4.39 ± *0.04*
Asp. versicolor	1.61 ± *0.11*	2.33 ± *0.17*	3.04 ± *0.03*	3.66 ± *0.04*	4.41 ± *0.13*
C. cladosporoides	1.64 ± *0.11*	2.30 ± *0.06*	3.07 ± *0.07*	3.69 ± *0.11*	4.34 ± *0.06*
S. chartarum	1.56 ± *0.11*	2.16 ± *0.06*	3.16 ± *0.03*	3.61 ± *0.06*	4.43 ± *0.10*

Note: *sd* = standard deviation.

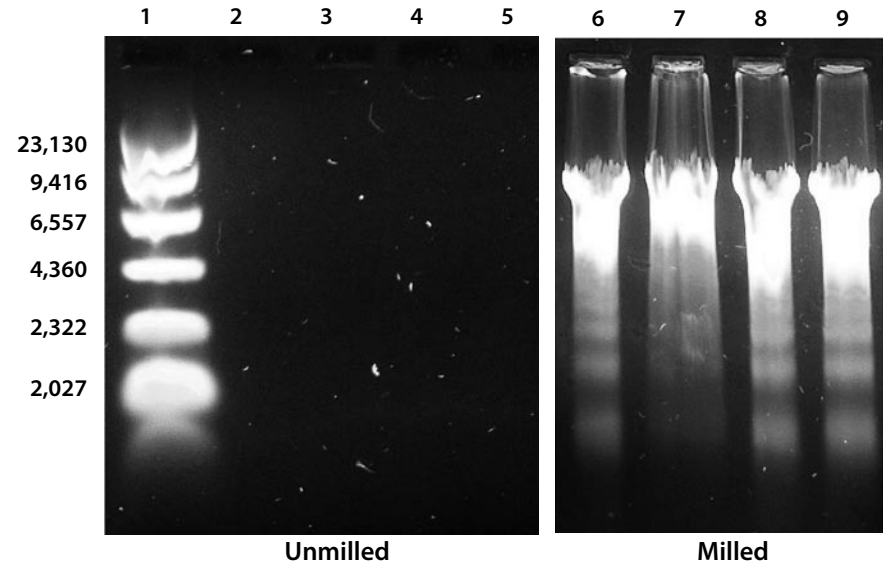


Figure 1. Gel electrophoresis of *Alternaria alternata* (lanes 2,6), *Aspergillus versicolor* (lanes 3,7), *Cladosporium cladosporoides* (lanes 4,8), and *Stachybotrys chartarum* (lanes 5,9), unmilled and milled DNA extractions

Note: A DNA ladder (lane 1) provides size in base pairs, which are specified on the left.

resulted in specific amplification (+) of the target species, while the negative control resulted in no reaction at all (–).

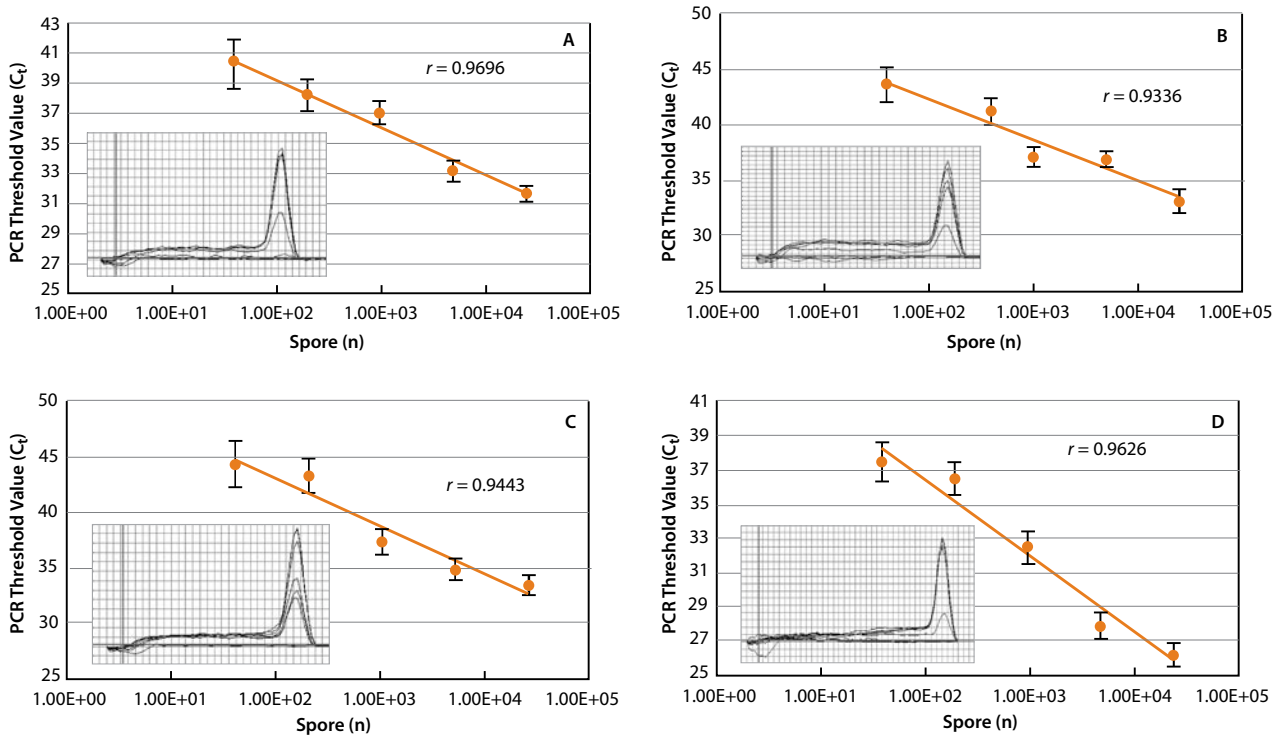
The qPCR analyses of *Alt. alternata, Asp. versicolor, C. cladosporoides*, and *S. chartarum* are presented in Figure 2 (A–D). The curves represent the results of three experiments; the Y-axis (C_t) is the cycle in which SYBR Green I fluorescence was detectable. The *r* value is included for each result, and the spore number (X-axis) indicates the number of spores used for genomic DNA preparation. For all fungi, as few as 40 spores and up to as many as 25,000 spores were detectable by qPCR assay; the average *r* was 0.95. The melt curve indicates that a single PCR product was generated in each qPCR assay; all peaks align, and there is no shift from the center characteristic of inefficient PCR and nonspecific amplification of multiple targets.

Table 2. Primer specificity in quantitative polymerase chain reaction

Organism Tested	Specificity Results for Each Organism			
	Alt. alternata	*Asp. versicolor*	*C. cladosporoides*	*S. chartarum*
Alt. alternata	+	–	–	–
Asp. versicolor	–	+	–	–
C. cladosporoides	–	–	+	–
S. chartarum	–	–	–	+
No DNA	–	–	–	–
Specific DNA	+	+	+	+

\+ indicates positive specificity result from qPCR analysis
– indicates negative specificity result from qPCR analysis

Figure 2. Quantitative polymerase chain reaction (qPCR) detection of *Alternaria alternata* (A), *Aspergillus versicolor* (B), *Cladosporium cladosporoides* (C), and *Stachybotrys chartarum* (D) spore preparations



Note: The quantitative linear regression represents the results of three experiments. The C_t value is the cycle threshold at which SYBR Green I fluorescence was detectable. The *r* value for each line is included. Spore number indicates the number of spores used in the DNA preparation. A melt curve (insert) represents the release of fluorescence upon melting of qPCR product: the lowest peak represents fluorescence for 40 spores and the highest peak represents fluorescence for 25,000 spores.

Discussion

The test organisms were field isolates of *Alternaria alternata, Aspergillus versicolor, Cladosporium cladosporoides,* and *Stachybotrys chartarum*. Each genus has been identified as a significant agent of human disease associated with allergy and asthma.[25-27] These fungi can persist as mixed populations in carpet and are very difficult to identify by morphological approaches alone.

Although the development of molecular technologies has expedited identification of different fungi, a single consensus approach is lacking. Various protocols are available for DNA isolation from fungi spores, but they entail cumbersome sonification steps and toxic chemical phenol-chloroform extractions. Besides being very time-consuming, these methods also result in DNA that produces inferior PCR.[28-30] One early study demonstrated that DNA was released from filamentous fungi hyphae after glass bead milling, but this process proved unsuccessful when disrupting spores.[31]

The advent of commercially available DNA isolation kits has reduced the cumbersome nature of earlier methods, but it has not overcome the adherent difficulties in extracting genomic DNA from recalcitrant structures such as spores. We overcame these difficulties through the modifications defined in this study, producing high-quality genomic DNA, which led to specifically amplified product in the qPCR assays. Furthermore, our use of the multi-copy rRNA region increased the sensitivity of qPCR detection because this region in fungi can have copy numbers of 100 or more.[32-37]

Detection of fungi contamination in the built environment remains problematic, because fungi inhabit surfaces such as carpet that becomes dirty. This factor makes genomic DNA extraction difficult, which in turn often leads to inferior analytical results. Our study demonstrates that milling spores in lysis buffer and removing cell debris by filtration provides high-molecular-weight DNA, which we could then use in qPCR assays for quick and accurate quantitation of fungi from carpet. Moreover, our results were reproducible. The high reproducibility of our results indicates that this method may be used to reliably identify fungi contamination in occupied spaces.

We believe that researchers and commercial firms interested in using qPCR for identification of fungi in the built environment can adopt the principles we describe here. We look forward to working with any interested parties in developing these procedures further.

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