USING PCR TO DETECT INDOOR FUNGI

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ABSTRACT
This research attempts to develop a molecular bioassay that uses a Polymerase Chain Reaction (PCR) technique for detecting fungi commonly found in an indoor environment. During sample preparation, the method of bead-beating was identified as the most effective way for spore breakage and fungal DNA release. In the process of assay development, a fungal primer set, FF2/FR1, was designed and tested with DNA from human, rats, mice, bacteria, pollens and six fungal species. The results based on the crude extracts indicated that the primer set successfully amplified the fungal DNA without any cross-amplification with non-fungal DNA. In addition, higher amplification efficiencies were achieved using 20% nutrient media, rather than water, as the process solution. This PCR assay has a sensitivity of detecting low levels of fungi (2 fungal spores per reaction).

INDEX TERMS
Fungi, Indoor air quality, Polymerase chain reaction (PCR), Exposure assessment, Allergies and asthma

INTRODUCTION
Biological hazards in an indoor environment are diverse. Reported problems have ranged from allergic and infectious diseases to nonspecific “sick building symptoms” such as headaches, fatigue, and chest tightness. According to the annual census of NIOSH health hazard evaluations, the proportion of workers’ requests related to indoor air quality in non-industrial environments has increased over the years, from 2% in 1980 up to 65% in recent years, and the costs are estimated at tens of billions of dollars per year (NORA, 1996). The conventional approach for assessing worker exposure to bioaerosols is to take samples and then perform laboratory analyses, which could be directly counting the organisms, indirectly counting colony-forming units, or using an immunoassay to detect the presence of specific biological agents. Although these methods may provide reasonably adequate assessment of bioaerosol concentration, the process of assessing exposure has been hampered by the lack of measurement techniques with near real-time, on-site, and high-sensitivity features. The present study is to establish and examine the applicability of a convenient, rapid, sensitive, and fungi-specific molecular detection system that uses polymerase chain reaction (PCR) to amplify DNA from the common indoor fungi without any cross-amplification with DNA from non-fungal organisms. This system would be useful in quickly screening low levels of airborne fungi in an environment and allow industrial hygienists or health professionals to rapidly determine the seriousness of fungal contamination and make investigation plans. In addition, this method has the advantage of monitoring for culturable fungal spores as well as non-culturable fungal substances that may not be viable but could be toxic or allergic.

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MATERIALS AND METHODS

PCR

PCR is a rapid and simple technique for specific amplification of a particular segment of DNA. Normally the amplification occurs as long as the segment is present in the sample regardless of the culturability or viability of the microorganisms. As in other cells, ribosomal RNA (rRNA) genes in a microbial cell are both highly conserved and present in multiple copies, thus making them popular targets for gene amplification and molecular analysis (Lee and Taylor, 1990; Einsele, Hebart, Roller, *et al.*, 1997). PCR techniques based on rRNA genes have been widely used in developing sensitive systems for the detection of microorganisms (White, Bruns, Lee, *et al*., 1990).

Test fungi selection and PCR primer design

Six fungal species, including *Alternaria chlamydospora*, *Aspergillus flavus*, *Candida famata*, *Cladosporium fermentans*, *Penicillium chrysogenum*, and *Stachybotrys chartarum*, were selected to represent the molds (except *Ca. famata*) commonly found in a problematic indoor environment (Godish, 1995). A PCR method without any cross-amplification with DNA from other common indoor organisms (i.e., bacteria, pollen, mite, algae, rat, mouse and human) was developed. The fungal forward-primer FF2 (5'-GGTTCTATTTTGTTGGTTTCTA-3'), and reverse-primer FR1 (5'- CTCTCAATCTGTCAATCCTTATT-3') were designed from the18S rRNA gene by selecting sequences homologous among the fungi, but not homologous with the other species. Although mite and algae were examined in their sequences during the primer design, they were not included in the actual experimental tests, because their sequences contain >10 mismatched bases with the designed primers and were thought not to be conducive to cross-amplification. Since fungal genetic characteristics are closer to plants than to bacteria, six types of plant pollen from cottonwood, red oak, ragweed, sagebrush, bentgrass, and Bermuda grass were selected to test the specificity of the fungal primers and only *Escherichia coli* was selected to represent the prokaryote in this test.

Spore breakage and DNA release

Fungal cultures were grown and spores were harvested. Several methods, including freeze-thaw, French press, freeze press, and bead-beating, were examined for the release of fungal DNA. The bead-beating method, by vigorously colliding cells with the 0.1-mm zirconia/silica beads, reached nearly 100% spore breakage (Biospec products, Bartlesville, OK) and, thus, was selected for breaking up fungal spores, bacteria, and pollens. Percentage of cell breakage was calculated by microscopically counting the cells with a haemocytometer before and after the treatment. A vortex was also used for bead-beating with a efficiency of 85-100%. Because it is smaller and lighter, it could be an alternative when using the assay in the field DNA from rat liver, BALB/c-3T3 cells (mouse) and human peripheral blood lymphocytes were extracted using the standard phenol/chloroform method (Sambrook, Fritsch, and Maniatis, 1989).

Fungal spores collected from the culture plates were counted under a microscope and then diluted serially from 10^6 to 10^2 spores/ml with 20% nutrient media (nutrient broth - beef extract, peptone, and salt). A 0.1 ml of sample was directly added into a 0.5 ml micro-tube containing approximately the same volume of beads and homogenized for 3 min, then heated in a boiling water bath for 5-10 min to inactivate released nucleases. The samples were cooled to room temperature and spun for 2-5 s in a micro-centrifuge to discard cell debris. The supernatants were directly used as templates for PCR.

Pollen and bacterial DNA were also released with a bead-beating technique using the same preparation steps as described above.

In parallel to the above study design, a protocol using a different preparation procedure (homogenization first rather than dilution first) and a different process solution (pure water rather than 20% nutrient media) were also followed to investigate the effects of PCR amplification efficiency on the procedure and the process solution.

PCR amplification and gel electrophoresis

Various supernatants (2 μl each) or DNA (200 ng) from human, rats or BALB/c-3T3 cells were amplified with each primer (10 pmol), Taq DNA polymerase (1.25 U), dNTPs (200 μM), and $MgCl_2$ (1.5 mM) in 50 μl of reaction buffer using a Thermal Cycler (Model 480, Perkin-Elmer Cetus, Foster City, CA). The conditions for PCR were as follows: initial denaturation of DNA at 95°C for 3 min and then 35 cycles of three-step PCR amplifications consisting of denaturation at 94°C for 1 min, primer reannealing at 52°C for 1 min, and extension at 72°C for 2 min. The samples were subjected to an additional extension at 72°C for 10 min at the end of the amplification cycles. Ten microliters of PCR products, mixed with loading buffer, were loaded on a 2.0% agarose : Nusieve GTG agarose gel (1:1) and electrophoresed with 1x TAE buffer at 8 V/cm. The gels were stained with ethidium bromide (0.5 μg/ml) in TAE for 10 min and visualized under UV light using an Eagle Eye II image system and photographed.

To investigate effects of fungal mixtures on the assay performance, various fungal spores were mixed in equal ratios to give similar total spore count (7.5 x 10^4 spores/ml) as that of individual spores and bead-beaten to release mixtures of spore DNA equivalents. These samples were prepared, amplified, and analyzed as described above.

RESULTS

Fungal specificity of primer pairs

To determine whether cross-amplification would occur, fungal, plant, human, rat, mouse, and bacterial DNA were broadly tested using the FF2/FR1 primer set. Figure 1 shows that this primer pair could successfully amplify the DNA of all fungal samples and yield an amplicon of 425 bp without showing any cross-amplification with non-fungal DNA. The amplified products were directly sequenced and the results indicated that the fragments from fungal samples indeed matched fungal DNA sequences.

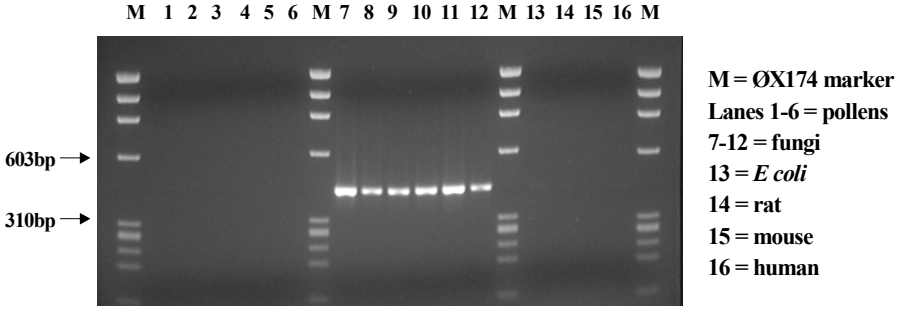

Figure 1. PCR products amplified by primers FF2/FR1 from various sources of DNA

Effects of procedure in spore preparation

In this study, the dilution-first and homogenization-first procedures were compared using 20% nutrient media. The amplified bands were observed at as low as 2 spore DNA equivalents/reaction using the dilution-first procedure, while the homogenization-first procedure could show amplified bands even at 0.2 spore DNA equivalent/reaction with 20% nutrient media.

Effects of process solution

The amplification efficiency in water or 20% nutrient media was measured using the homogenization-first procedure to determine whether PCR would be hindered when DNA from disrupted spores was amplified directly. After 10^6 fungal spores/ml were disrupted and diluted tenfold serially, PCR amplification was performed to determine the efficiency of the assay system. Positive PCR bands were shown clearly at 2 x 10^3 to 0.2 fungal spore DNA/reaction (equivalent to 10^6 to 10^2 spores/ml in concentration) and the amplification efficiency increased with the concentration (Table 1), except for *Alternaria* and *Stachybotrys* in which the efficiencies decreased at concentrations higher than 2 x 10^3 spore DNA/reaction. Overall, a higher amplification efficiency was observed when 20% nutrient media was used to replace water as the cell breakage and dilution solution for all samples.

Table 1. Intensity of PCR Amplification in Different Breakage Solutions

Fungal spore	Spore DNA/reaction in medium					Spore DNA/reaction in water				
	0.2	2	20	200	2000	0.2	2	20	200	2000
Alternaria chamydopora	++	+++	++++	+++++	+++	+	++	+++	++++	++
Aspergillus flavus	+	++	+++	++++	+++++	-	+	++	+++	++++
Candida famata	+	++	+++	++++	+++++	-	+	++	+++	++++
Cladosporium fermentans	+	++	+++	++++	+++++	-	+	++	+++	++++
Penicillium chrysogenum	+	++	+++	++++	+++++	-	+	++	+++	++++
Stachybotrys chartarum	+	++	+++	++++	+++	-	+	++	+++	+++

-, no amplification; +, very weak signal; ++, weak signal; +++, moderate signal; ++++, strong signal; +++++, very strong signal

Effects of mixtures

PCR amplification from crude extract of mixtures of spores produced an amplification product of the expected size similar to the PCR product from individual spores (Figure 2). The PCR products were semi-quantitated by analysis of gel image using Image Quant 3.3 and molecular weight markers for calibration. The amplified products were in the range of 70.61 to 93.77 ng of DNA/band, with an average of 81.90 ± 7.27 (s.d) corresponding to approximately 150 spore DNA equivalents/reaction in PCR assay. Although this is a preliminary study on 3 different fungi mixed in equal ratios, the results were reproducible with amplified products within the same range.

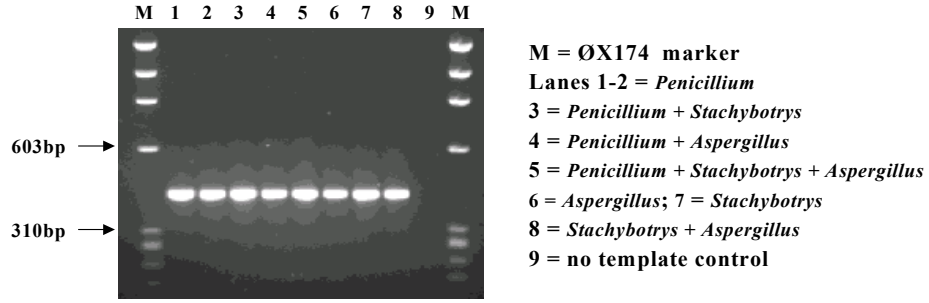

Figure 2. PCR products amplified by primers FF2/FR1 from various mixtures of spore DNA

DISCUSSION

A microbiological assay system has been established in our laboratory, which has the potential to become a screening tool to detect total airborne fungi in the indoor environment. This system includes a bead-beating process for breaking up the spores in the sample and a fungi-specific primer pair (FF2/FR1) to amplify the fungal DNA retrieved from spore breakage.

For environmental monitoring, it is important to exclude false positives that could result from the lack of specificity of the assay system. Real-life samples from the air, water or soil usually contain some degree of biological contaminants associated with human, rat, mouse, mite, algae, pollen or bacterial cells which may have potential for cross-amplification with fungi when applying PCR analysis. Most PCR assay systems reported in the literature have focused primarily on clinically relevant fungi and, thus, considered mainly cross-amplification with human and bacterial DNA (Makimura, Murayama and Yamaguchi, 1994; Einsele, Hebart, Roller, *et al.*, 1997), as well as plant DNA, (Kappe, Fauser, Okeke, *et al.*,1996; Kappe, Okeke, Fauser, *et al.*, 1998) but not other biological contaminants. Our previous study on examining other "universal" fungal primers (Zhou, Wong, Ong, *et al.*, 2000) demonstrated that the primer pair, FF2/FR1 had no cross-amplification with rat and mouse DNA, and has more than 10 mismatched bases with the sequences of mite and algae. In addition, this primer set may be a suitable fungi-specific primer set for screening fungal presence in a setting other than a problematic indoor workplace, e.g., a hospital environment as determined by either complete homology or 1 base mismatch with 18 medically relevant fungi.

In this study, the supernatants were directly used because it would make the assay protocol more convenient and practical if cell-disrupted crude extract from the samples could be used directly for PCR without DNA purification. However, fungi supernatant may contain contaminants that could affect PCR amplification. When the nutrient media was used as the processing solution, proteins or other substances in the nutrient media might have increased PCR efficiency by preventing released fungal DNA from nonspecifically sticking to the beads as well as combining with inhibition-causing substances to help stabilize the Taq DNA polymerase. Practically speaking, a procedure of serial dilution of the crude DNA release after breakage might be needed for samples of high fungal concentrations (e.g., in an agricultural or other industrial environments) to attenuate the inhibition effect. For the samples collected from a nonindustrial indoor environment, the spore counts without dilution would be similar to the situation used in the dilution-first procedures and the sensitivity of 2 fungal spores/reaction would be a more proper representative of this assay system. The value is similar to that reported by Haugland and Heckman (1999) in which they describe a TaqMan^{TM} system able to measure as low as 2 conidia/reaction of *S. chartarum*.

The simple three-fungi mixture study on PCR assay showed amplification products within a close range. However, the study was performed for mixtures in equal ratios. It is well documented that PCR bias due to many factors exists (Farrelly, Rainey, and Stackebrandt, 1995; Suzuki and Giovannoni, 1996; Polz and Cavanaugh, 1998). These include genome size, rDNA operon numbers, PCR selection, PCR drift, template saturation, etc. For most fungi, the detailed study of the overall genome is not known. It will be important to explore PCR bias from mixed template PCR reactions to interpret PCR-based results in a quantitative measure.

In summary, the designed primer set, FF2/FR1, has been tested to be fungus-specific and this PCR assay has a sensitivity of detecting low levels (2 spores) of fungi in a reasonable time period (5-6 h). This technique may be useful in estimating fungal bio-mass in an

environmental sample in general and have particular value in investigations of indoor air quality where exposure levels are low and adverse health effects are due to allergic and toxic reactions. It is, however, essential to test spiked environmental samples in determining the actual limits of detection as well as the effects of environmental inhibitors to the PCR prior to any actual field applications.

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