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Development of a fungus-specific PCR assay for detecting low-level fungi in an indoor environment

G. Zhou,[1,2] W.-Z. Whong,[1] T. Ong[1] and B. Chen[1]*

[1]*Health Effects Laboratory Division, National Institute for Occupational Safety and Health (NIOSH), Centers for Disease Control and Prevention, Morgantown, WV 26505–2845, USA,* [2]*Department of Toxicology, West China University of Medical Sciences, Chengdu, Sichuan, China*

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A fungus-specific PCR assay using only one primer set has been developed for detecting indoor fungi. Four fungal primer sets, NS3/NS4, NS5/NS6, FF1/FR1 and FF2/FR1, were tested with DNA from humans, rats, mice, bacteria, pollens and six commonly found fungal species (*Alternaria chamydospora, Aspergillus flavus, Candida famata, Cladosporium fermentans, Penicillium chrycogenum* and *Stachybotrys chartarum*). Results indicated that, although all four primer sets could amplify the fungal DNA, only FF2/FR1 demonstrated no cross-amplification with non-fungal DNA. In addition, these amplified fragments were sequenced to ensure that they indeed matched known fungal DNA sequences. Furthermore, besides the tested fungi, eighteen more genera of fungal sequences were examined and found to match the FF2/FR1. Here, the method of bead-beating was identified as the most effective way for spore breakage and fungal DNA release. The PCR amplification efficiency and potential inhibition were examined using different process solutions and preparation procedures. It was found that, when using 20% nutrient media and homogenization-first procedure, a higher amplification efficiency with less inhibition was achieved. Although positive bands were observed at 0·2 fungal spore/reaction using the homogenization-first procedure, the sensitivity of this assay would be two fungal spores/reaction for environmental samples.

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KEYWORDS: fungus-specific primer, indoor fungi, 18 S rRNA, PCR, indoor air quality.

INTRODUCTION

It has been estimated that 4% of all Americans are afflicted with asthma, with or without allergic rhinitis, and 7% have allergic rhinitis alone.[1] In a large number of these cases, inhalant allergens, particularly molds, are involved and are one of the most common triggers of human atopic diseases, such as allergic rhinitis. Fungi exist both indoors and outdoors. Freezing and/ or dry weather can damage fungi and reduce the spore counts on outdoor samples, but the conditions indoors may be very hospitable to fungal growth non-seasonally.[1] Such an indoor environment, with the potential of prolonged and continuous exposure of sensitive individuals to the airborne allergens, increases the risk of making these respiratory diseases more severe.

It would be very useful if the initial stage of an environmental survey were to include the ability to quickly screen and roughly estimate fungi in the environment, because this would allow one to rapidly determine the seriousness of fungal infestation, to

* Author to whom all correspondence should be addressed at: Exposure Assessment Branch, Health Effects Laboratory Division, NIOSH, 1095 Willowdale Road, Morgantown, WV 26505, USA. Tel: +304 285 6148; Fax: +304 285 6041; E-mail: bdc4@cdc.gov

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make or modify investigation plans and to save money and time. Conventional laboratory detection of fungi often requires culture isolation and relies on a variety of morphological and physiological tests.[2–6] This process is tedious and time-consuming, taking days to weeks. In addition, culturable fungal spores do not account for all the fungi-related diseases, and non-culturable spores could still be viable and allergenic and cause problems.[7] Therefore, environmental monitoring of microorganisms to detect potential sources of pathogens for preventive public health and epidemiological purposes does require a rapid, sensitive and reliable approach which can detect microbes whether they are culturable or non-culturable.

PCR is a rapid and sensitive 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, therefore making them popular targets for gene amplification and molecular analysis.[5,8–10] PCR techniques based on rRNA genes have been used widely in developing sensitive systems for the detection of micro-organisms.

For the detection of fungi, research studies focus primarily on clinically relevant pathogenic fungi and most developed assay systems concentrate on qualitative detection or diagnosis of specific fungi. Although some 'universal' primers targeted to the small ribosomal subunit gene (18 S rRNA) have been described, these systems are not suitable for environmental monitoring because of cross-amplification and/or lack of coverage of common indoor fungi (e.g. *Alternateria*).[3,11–13] White *et al.*[13] described some universal primers that can amplify fungal DNA as well as some plant and vertebrate DNA. Makimura *et al.*[12] reported a universal primer system which amplified DNA from 78 strains of 25 medically relevant fungi but not *Alternateria*.[3] Kappe *et al.*[3,11] developed several universal primer systems which amplified up to 65 of 67 species of pathogenic fungal DNA. These primer pairs, however, also amplified some plant DNA (e.g. *Physcomitrella patens* and *Brassica napus*) and produced the same size fragments as fungi. It appears that some fungi-specific primer systems may exist for detecting fungi present in certain specific clinical areas; however, there is a need for the development of a fungi-specific PCR assay for detecting the common fungi in indoor environments.

The present study attempts to establish a convenient, rapid, sensitive and fungus-specific detection system which uses PCR to amplify DNA from the common fungi without any cross-amplification with DNA from prokaryote (bacteria) and other eukaryotes (pollen, rat, mouse and human). It is intended that this system could be utilized to detect low levels of fungi for environmental monitoring.

MATERIALS AND METHODS

Cultures of fungi, pollens and bacteria

Six commonly found fungi were selected for the study. Five fungi, *Alternaria chamydospora* (28045), *Aspergillus flavus* (16883), *Candida famata* (10539), *Cladosporium fermentans* (34011) and *Penicillium chrysogenum* (10106), were obtained from ATCC (American Type Culture Collection, Rockville, MD, USA) and cultured separately on agar plates containing Difco 0549, 0338, 0711, or 0113 nutrient media (Difco Laboratories, Detroit, MI, USA). *Stachybotrys chartarum* was provided by Dr Pengfei Gao (NIOSH, Morgantown, WV, USA) and cultured using the method reported by Sorenson *et al.*[14] All, except *C. famata*, were selected to represent commonly observed species of fungi in a problematic indoor environment.[7] Since fungal genetic characteristics are closer to pollen than to bacteria, six types of pollen (*Populus deltiodes*, *Quercus rubra*, *Ambrosia trifida*, *Artemisia tridentata*, *Agrostis alba* and *Cynodon dactylon*) from cottonwood, red oak, ragweed, sagebrush, bentgrass, and Bermuda grass, respectively, were selected to test the specificity of the fungal primers. They were purchased from Sigma (St Louis, MO, USA). Only one type of bacteria (*Escherichia coli*; 11775) was selected to represent the prokaryote in this test and it was obtained from ATCC and cultured on agar plates containing Difco 0003 nutrient media prior to harvest.

Spore breakage

Cultures were routinely grown for 7–10 days at room temperature, and the spores were harvested by gently rolling a moistened, sterile cotton swab over the surface of the colonies and then suspended in a liquid solution. To determine the breakage efficiencies of fungal spores, several methods, including the freeze press, French press, freeze/thaw and bead beater, were compared in the preliminary study. *S. chartarum* spores were selected for this comparison because their sizes were relatively big and their cell walls were hard to break. Percentage of cell breakage was calculated by counting the cells under a microscope with a hemacytometer before and after treatment. In

the case of bacteria, pollens and other fungi, only the bead-beating method was used. Because bacteria and pollens are mainly used for examining potential cross-amplification of each primer pair, the breakage efficiencies were not quantitatively determined.

DNA release and/or extraction

Besides pollen and bacteria, humans, mice and rats were also selected to test the specificity of the primer pairs. DNA from rat liver, BALB/c-3T3 cells and human peripheral blood lymphocytes were extracted using the standard phenol/chloroform method.[15]

Based on the spore breakage efficiencies obtained from the preliminary study, the method of bead-beating was selected for the release of fungal DNA by vigorously colliding cells with 0·1-mm zirconia/silica beads (Biospec products, Inc., Bartlesville, OK, USA). Two different procedures, homogenization-first and dilution-first, and two different solutions, water and 20% (w/v) nutrient media, were used to study the effects of preparation procedures on the PCR amplification.

Fungal spores collected from the culture plates were suspended in water or 20% nutrient media (nutrient broth–beef extract, peptone and salt) (Oxoid, Basingstoke, Hampshire, UK) and counted under a microscope. For the homogenization-first procedure, 0·1 ml of sample was directly added into a 0·5 ml Eppendorf tube containing approximately the same volume of beads. The tube was either fastened on a vortex by rubber bands and vortexed vigorously for 3–5 min or put into a Mini-Bead Beater (Biospec products, Inc.) 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 an Eppendorf micro-centrifuge to collect condensate and deposit cell debris. The supernatant fluid was serially diluted with water or 20% nutrient media from 10^7 to 10^2 spores/ml (note that this unit is spore equivalent since spores were actually disrupted in this case). For the dilution-first procedure, after cell counting, the samples were diluted serially from 10^6 to 10^2 spores/ml with water or 20% nutrient media, then 0·1 ml of the diluted samples were separately homogenized, heated, cooled, and centrifuged similar to those described in the homogenization-first procedure. In each case, 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 in the homogenization-first procedure except that only the solution of 20% nutrient media was used.

PCR primer design

The 18 S or 16 S rRNA gene sequences of fungi, humans, mice, rats, mite, algae, pollens and bacteria were retrieved from the NCBI GenBank database [http://www.ncbi.nlm.nih.gov/Entrez]. The fungal forward-primers FF1 (5′-GTTAAAAAGCTCGTAGTTG-AAC-3′), FF2 (5′-GGTTCTATTTTGTTGGTTTCTA-3′), and reverse-primer FR1 (5′-CTCTCAATCTGTCAA-TCCTTATT-3′) were designed in the laboratory from the 18 S rRNA gene (Fig. 1) by selecting sequences homologous among the fungi, but not homologous with other organisms (i.e. humans, rats, mice, mite, algae, bacteria and pollens). Note that, 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.

The specificity of our fungi primers was examined by comparing with published 'universal' fungi primers such as: NS3 (5′-GCAAGTCTGGTGCCAGCAGCC-3′)/NS4 (5′-CTTCCGTCAATTCCTTTAAG-3′) and NS5 (5′-AACTTAAAGGAATTGACGGAAG-3′)/NS6 (5′-GCATCACAGACCTGTTATTGCCTC-3′).[13] All the primers used in this study were synthesized by Cruachem Inc. (Aston, PA, USA).

PCR amplification

Various supernatants (2 μl each) or DNA (200 ng) from human, rat or BALB/c-3T3 cells were amplified with each primer (10 pmol), *Taq* DNA polymerase (1·25 U; Promega, Madison, WI, USA), dNTPs (200 μM; Promega), and $MgCl_2$ (1·5 mM; Promega) in 50 μl of reaction buffer using a DNA Thermal Cycler (Model 480, Perkin-Elmer Cetus, Foster City, CA, USA). 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 NS3/NS4, FF1/FR1, FF2/FR1) or 56°C (for NS5/NS6) 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.

Gel electrophoresis

Ten microlitres of PCR products, mixed with loading buffer, were loaded on a 2·0% (w/v) agarose: Nusieve

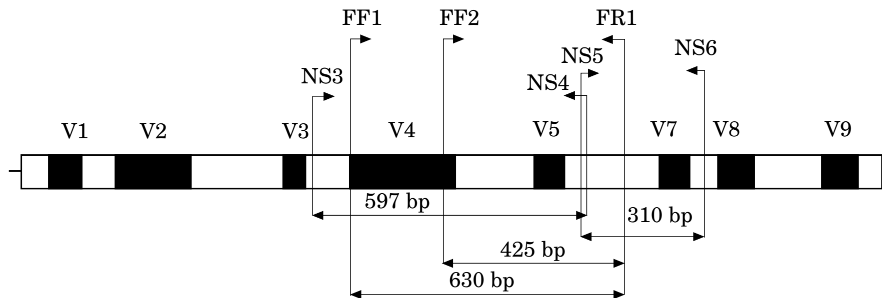

Fig. 1. Map of the 18 S rRNA gene (adapted from Kappe *et al.*)[3] with highly conserved regions (□) and variable regions V1 to V9 (■), the positions of primers and the sizes of amplified fragments are indicated.

GTG agarose (Intermountain Scientific Corp., Rockland, ME, USA) gel (1:1) and electrophoresed with 1 × 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 U.V. light using an Eagle Eye II image system (Stratagene, La Jolla, CA, USA) and photographed.

DNA sequencing

PCR products were purified to remove excess primer using microconcentrators (Amicon, Inc., Beverly, MA, USA), and then directly sequenced with the Dye-Deoxy Terminator Cycle Sequencing kit in an automated DNA Sequencer (Model 373A, Applied Biosystems, Foster City, CA, USA).

RESULTS

Breakage efficiency of fungal spores

The breakage efficiency of fungal spores was studied using various methods with *S. chartarum* spores. The freeze-thaw method achieved less than 2% spore disruption, while 74% and 81% efficiencies were observed with the French press and freeze press methods, respectively. The bead-beating method using the Mini-Bead Beater resulted in the most efficient spore breakage with 0·5-mm glass beads yielding a 91% breakage efficiency and 0·1-mm Zirconia/Silica beads reaching 100% spore breakage. This 100% efficiency applied to all the fungal species used in this study and, therefore, this method was selected throughout the study. Lower efficiencies (85–100%, depending on the species) were obtained when bead-beating was applied in a regular vortex, rather than the Mini-Bead Beater. No difference was observed in breakage efficiency between the use of water and 20% nutrient media when the Mini-Bead Beater was used.

Fungal specificity of primer pairs

To determine whether cross-amplification would occur, fungal, plant, human, rat, mouse and bacterial DNA were broadly tested using all four primer sets. The results based on the homogenization-first procedures with 20% nutrient media are shown in Table 1. Although every tested primer set could successfully amplify the DNA of all fungal samples used in this study, FF2/FR1 primer pair is the only set that did not show any degrees of cross-amplification (Fig. 2). NS3/NS4 amplified all types of DNA except *A. alba*, *C. dactylon* and *P. deltiodes*, but a different size fragment was observed with bacterial DNA (Fig. 3). NS5/NS6, although avoiding cross-amplification with *E. coli*, had broad cross-amplification with mouse, rat and four types of pollen DNA (Table 1). FF1/FR1 appeared to be a good candidate for a fungus-specific primer pair with no positive PCR-amplified bands being detected in human, rat, mouse, bacteria and five types of the pollen DNA. However, at a high concentration of *A. trifida* (>10^5 pollen grains/ml), positive PCR-amplified bands resulted as shown in Table 1.

The products amplified by primer pairs FF1/FR1 and FF2/FR1 were directly sequenced. The results indicated that the fragments from fungal samples indeed matched fungal DNA sequences. Since a band was observed with primers FF1/FR1 in a pollen sample (*A. trifida*), these products were also sequenced. The result indicated a match with the plant DNA sequences.

Effects of procedure in spore preparation

Here, the dilution-first and homogenization-first procedures were compared using 20% nutrient media. The amplified bands were observed at as low as two spore DNA/reaction using the dilution-first procedure, while the homogenization-first procedure could show

Table 1. The specificity of primer pairs

DNA sample	NS3/NS4	NS5/NS6	FF1/FR1	FF2/FR1
Human	+	−	−	−
Rat	+	+	−	−
BALB/c-3T3	+	+	−	−
Escherichia coli	+[a]	−	−	−
Fungi				
Alternaria chlamydopora	+	+	+	+
Aspergillus flavus	+	+	+	+
Candida famata	+	+	+	+
Cladosporium fermentans	+	+	+	+
Penicillium chrysogenum	+	+	+	+
Stachybotrys chartarum	+	+	+	+
Pollens				
Agrostis alba	−	−	−	−
Ambrosia trifida	+	+	+[b]	−
Artemisia tridentata	+	+	−	−
Cynodon dactylon	−	−	−	−
Populus deltiodes	−	+	−	−
Quercus rubra	+	+	−	−

[a] Different size fragment.
[b] Positive amplification only applies to pollen concentrations greater than 10^5 pollen grains/ml.
+, positive amplification; −, no amplification.

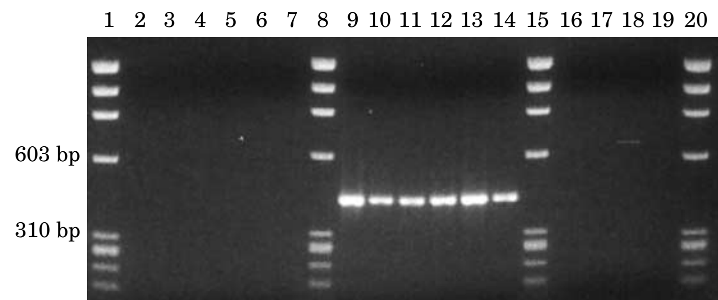

Fig. 2. PCR products amplified by primers FF2/FR1 from various sources of DNA: lanes 2–7, *Quercus rubra, Populus deltiodes, Cynodon dactylon, Artemisia tridentata, Ambrosia trifida* and *Agrosis alba*; lanes 9 – 14, *Stachybotrys chartarum, Penicillium chrysogenum, Cladosporium fermentans, Candida famata, Aspergillus flavus* and *Alternaria chamydospora*; lanes 16–19, *Escherichia coli*, Rat, BALB/c-3T3 and Human; lanes 1, 8, 15 and 20, molecular weight markers (φX174 RF DNA/*Hae*III fragments).

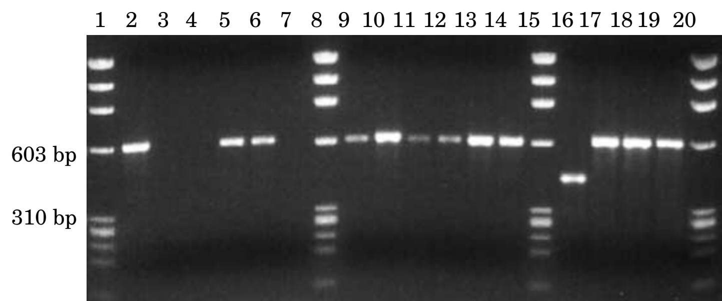

Fig. 3. PCR products amplified by primers NS3/NS4 from various sources of DNA: lanes 2–7, *Quercus rubra, Populus deltiodes, Cynodon dactylon, Artemisia tridentata, Ambrosia trifida* and *Agrosis alba*; lanes 9 – 14, *Stachybotrys chartarum, Penicillium chrysogenum, Cladosporium fermentans, Candida famata, Aspergillus flavus* and *Alternaria chamydospora*; lanes 16–19, *Escherichia coli*, Rat, BALB/c-3T3 and Human; lanes 1, 8, 15 and 20, molecular weight markers (φX174 RF DNA/*Hae*III fragments).

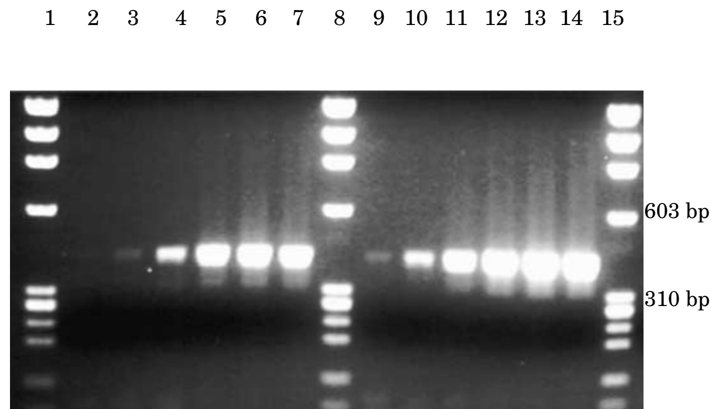

Fig. 4. Gel electrophoresis of PCR-amplified DNA with primers FF2/FR1 from different concentrations of DNA of *Cladosporium fermentans* in different spore preparation procedures: lanes 1, 8 and 15, molecular weight markers (φX174 RF DNA/*Hae*III fragments); lanes 2–7, in dilution-first procedure, 2×10^{-1}, 2×10^{0}, 2×10^{1}, 2×10^{2}, 2×10^{3} and 2×10^{4} spores/reaction; lanes 9–14, in homogenization-first procedure, 2×10^{-1}, 2×10^{0}, 2×10^{1}, 2×10^{2}, 2×10^{3} and 2×10^{4} spores/reaction.

amplified bands even at 0·2 spore DNA/reaction with 20% nutrient media (Fig. 4).

Effects of process solution

The amplification efficiencies in water or 20% nutrient media were measured using the homogenization-first procedure to determine whether PCR would be hindered when the disrupted supernatants were amplified directly. After 10^{7} 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 2×10^{4} to 0·2 fungal spore DNA/reaction (equivalent to 10^{7} to 10^{2} spores/ml in concentration) and the amplification efficiency increased with the concentration (Table 2 and Fig. 5), except for *Alternaria* and *Stachybotrys* in which the efficiencies decreased at concentrations higher than 2×10^{3} spore DNA/reaction (Table 2). A higher amplification efficiency was observed when 20% nutrient media was used to replace water as the cell breakage and dilution solution.

DISCUSSION

A microbiological assay system has been established in the laboratory which has the potential to become a screening tool to detect total airborne fungi in the environment. This system includes a bead-beating device for breaking up the cell walls of the spores in the sample and a fungi-specific primer pair (FF2/FR1) to amplify the fungal DNA retrieved from the cell breakage. Results from this study demonstrate that homogenization-first procedures using the sample mixed with 20% nutrient media could reduce potential inhibitory effects in the PCR assay and the detection limit can be better than two spore per reaction.

Unlike human, animal and bacterial cells, fungal spores have tough cell walls and ordinary cell lysis treatments, such as chemical digestion, freeze/thaw and freeze press, cannot effectively disrupt them and release DNA. The bead beater is a convenient, rapid and very effective device for disrupting fungal spores and yields high DNA recovery.[4,6,16–19] Our results showed that the Mini-Bead Beater achieved 100% breakage efficiency for the fungal spores used. Although the vortex has a slightly lower efficiency (85–100%) than the Mini-Bead Beater, it is much smaller, lighter and easier to transport and could be a good alternative when applying this assay for qualitative determination in the field.

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,[3,6,8,11,12,17,19–21] as well as plant DNA,[3,11,22] but not other biological contaminants. Our results on the universal fungal primers NS3/NS4 and NS5/NS6 showed cross-amplification to rat and mouse DNA,

Table 2. Intensity of PCR amplification in different breakage solutions

	Spore DNA/reaction in medium						Spore DNA/reaction in water					
	0·2	2	20	200	2000	20000	0·2	2	20	200	2000	20000
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.

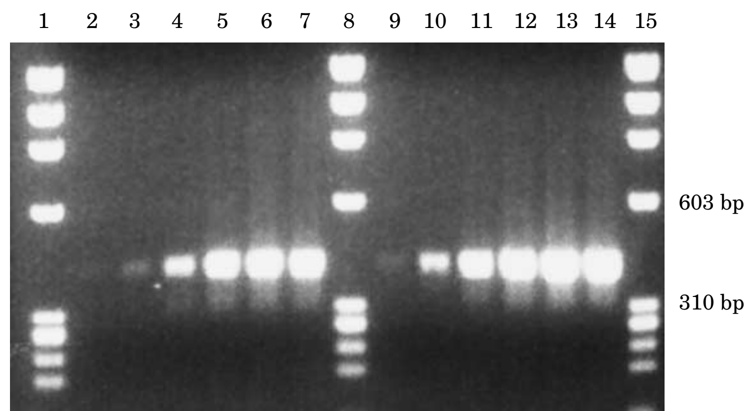

Fig. 5. 2·0% (w/v) agarose gel electrophoresis of PCR-amplified DNA with primers FF2/FR1 from different concentrations of DNA of *Penicillium chrysogenum* in water or nutrient media: lanes 1, 8 and 15, molecular weight markers (φX174 RF DNA/*Hae*III fragments); lanes 2–7, in water, 2×10^{-1}, 2×10^{0}, 2×10^{1}, 2×10^{2}, 2×10^{3} and 2×10^{4} spore DNA/reaction; lanes 9–14, in nutrient media, 2×10^{-1}, 2×10^{0}, 2×10^{1}, 2×10^{2}, 2×10^{3} and 2×10^{4} spore DNA/reaction.

indicating that they were not specific to fungi. This finding demonstrates that the cross-amplification with rat and mouse should not be omitted when the specificity of an assay system is tested. The same concern should apply to mite and algae as well. Here, the issue was indirectly resolved by designing the primer set, FF2/FR1, which has more than 10 mismatched bases with the sequences of mite and algae.

The first pair of primers, FF1/FR1, designed in the laboratory met almost all the fungi-specific requirements set up in this study, which include amplification of the DNA from six representatives of the most common indoor fungi but not the DNA from humans, rats, mice, bacteria or pollens. The only deficiency was its cross-amplification with the DNA of *A. trifida* at concentrations higher than 10^5 pollen grains/ml. To improve fungal specificity, the forward primer, FF1, was redesigned by shifting its location in the 18 S rRNA gene (Fig. 1) to yield a shorter amplicon (425 bp vs 630 bp resulted from FF1/FR1) and the new primer set, FF2/FR1, did successfully avoid the cross-amplification with *A. trifida*. Besides the tested fungi, 18 genera of medically relevant fungal sequences were retrieved from GeneBank and examined. The results indicated that primers FF2/FR1 match every single base with *Ajellomyces*, *Trichothecium*, *Paecilomyces*, *Microsporum*, *Coccidioides*, *Chrysosporium*, *Epidermophyton*, *Aureobasidium*, *Trichophyton*, *Saccharomyces*, *Blastomyces*, *Debaryomyces* and *Histoplasma*, and mismatch only one base with *Pneumocystis*, *Leptosphaeria*, *Basidiobolus*, *Graphium* and *Fusarium*. This suggests that the primer set FF2/FR1 may be a suitable fungi-specific primer set for screening fungal presence in a hospital environment.

Here, the supernatants were used directly. This is because the assay system would be more convenient and practical if cell-disrupted supernatant extracted from the samples could be used directly for PCR without DNA purification. However, fungi supernatant may contain chemicals which could affect PCR amplification. This phenomenon was investigated indirectly by comparing the PCR results from two solutions (water and 20% nutrient media) used. Results in Table 2 indicate that: (i) amplification efficiency is always higher when 20% nutrient media is used as the breakage solution compared to water; (ii) the efficiency increases with spore DNA concentration for *A. flavus*, *C. famata*, *C. fermentans* and *P. chrysogenum*; and (iii) for *A. chamydospora* and *S. chartarum*, the efficiency increases with spore DNA concentration at low concentration but not at concentrations higher than 2×10^3 spore DNA/reaction. These results suggest that some substances in the supernatant of *Alternaria* and *Stachybotrys* species might have inhibited *Taq* DNA polymerase activity, interfered with primer binding, or chelated magnesium and hindered the PCR amplifications and, as a result, broke the trend of stronger positive PCR amplified bands at higher spore concentrations, though the effects are insignificant at low concentrations. When the nutrient media was used as the solution, proteins or other substances in the nutrient media might have increased PCR efficiency by preventing released fungal DNA from non-specifically sticking to the beads as well as combining with inhibition-causing substances to help stabilize the *Taq* DNA polymerase.[23,24] Although nutrient media seems to be better in performance than water as the breakage solution, its mechanism could depend on the fungal

species, spore concentration, percent of the nutrient media, as well as other environmental factors and complete understanding requires further evaluation. 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. Although dilution can also reduce the sensitivity, the high sensitivity of the PCR system would not make it a problem.

The fungal PCR assay system based on the 18 S rRNA gene is extremely sensitive because a major rRNA gene has between 100 and 200 copies in lower eukaryotes (e.g. *Saccharomyces cerevisiae* has 140 18 S/28 S genes).[5,8–10] Although the results from literature have shown that 100 fg to 1 pg of fungal DNA or five to ten fungal cell/reaction[12,17,19,21,25] could be detected, reports that claim the capability of detecting DNA from a single cell containing only one genome[22,23,26–28] reveal the possibility of developing a fungi-detecting assay with a higher sensitivity. The system we have developed has the sensitivity of 0·2 spore/reaction with the homogenization-first procedure and two spores/reaction with the dilution-first procedure.

A higher sensitivity may be expected in the homogenization-first procedure since the released fungal DNA, rather than the intact spores as in the case of dilution-first procedure, were used in the serial dilution of samples. There are more copies of the fungal DNA than there are spores; therefore, there is a greater probability for a sample from the homogenization-first procedure to contain at least one copy of DNA available for PCR to result in a positive band. Different outcomes may be partially attributed to a higher proportion of beads relative to the number of spores in the dilution-first procedure. The increased bead surface on which the released DNA could be attached would subsequently leave less DNA available for PCR.

When applying this PCR assay to the samples collected from a non-industrial indoor environment, the sensitivity may not achieve 0·2 spore/reaction as shown in the homogenization-first procedure. Assuming that the average fungal spore level is less than 200 CFU/m^3 in concentration,[7] and a personal sampler is used for collecting aerosol on a filter at a flow rate of 2 l/min for 6 h,[29] the average total spore count on the filter would be less than 10^3 spores and the sample prepared in a 0·1-ml solution would contain less than 10^4 spores/ml in the average concentration, assuming a 25% of average spore culturability[30] and a 100% of spore recovery efficiency from the filter. In this case, the dilution-first procedure, rather than the homogenization-first procedure, is a more proper representative to mimic the preparation process for the sample and the sensitivity of the PCR assay system would be two fungal spores/reaction. This value is similar to that reported by Haugland *et al.*,[31] in which they describe a TaqMan™ system able to measure as low as two conidia/reaction of *S. chartarum*.

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