A Simple Multiplex Polymerase Chain Reaction Assay for the Identification of Four Environmentally Relevant Fungal Contaminants

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Abstract

Historically, identification of filamentous fungal (mold) species has been based on morphological characteristics, both macroscopic and microscopic. These methods have proven to be time consuming and inaccurate, necessitating the development of identification protocols that are rapid, sensitive, and precise. The polymerase chain reaction (PCR) has shown great promise in its ability to identify and quantify individual organisms from a mixed culture environment; however, the cost effectiveness of single organism PCR reactions is quickly becoming an issue. Our laboratory has developed a simple method to identify four mold species, *Stachybotrys chartarum*, *Aspergillus versicolor*, *Penicillium purpurogenum*, and *Cladosporium* spp. by performing multiplex PCR and distinguishing the different reaction products by their mobility during agarose gel electrophoresis. The amplified genes include the *β-Tubulin* gene from *Aspergillus versicolor*, the *Tri5* gene from *Stachybotrys chartarum*, and ribosomal sequences from both *Penicillium purpurogenum* and *Cladosporium* spp. This method was found to be both rapid and easy to perform while maintaining high sensitivity and specificity for characterizing isolates, even from a mixed culture.

1. Introduction

The indoor environment has become an important area of research in recent years. A central dynamic affecting the quality of the indoor environment is the control and removal of filamentous fungi (mold). It has been estimated that upwards of 40% of all homes in North America contain mold growth, while in Northern Europe the proportion is between 20-40%. (Brunekreef et al., 1989; Nielsen, 2003) Currently, of these mold species, only a small percentage is implicated in adverse health effects, however, increased awareness and additional research may show that adverse health issues are related to a broader range of fungal species. Filamentous fungi such as *Stachybotrys chartarum, Penicillium purpurogenum, Aspergillus versicolor,* and *Cladosporium* spp. are organisms that have been isolated from unhealthy buildings and are potentially associated with negative health effects in humans (Meklin et al., 2004; Vesper et al., 2004). These health effects may include itchy eyes, stuffy nose, headache, fatigue, and in severe cases idiopathic pulmonary hemosiderosis (IPH) in infants (Kuhn and Ghannoum, 2003; Mahmoudi and Gershwin, 2000; Vesper et al., 2000; Dearborn et al., 1999; Gent et al., 2002; Etzel, 2003; Andersen et al., 2002). In order to properly assess the fungal exposure to building occupants, and implement and carry out an effective remediation strategy, it is imperative that fungal screening and identification is carried out that is rapid, cost effective, and unambiguous.

Historically, fungal isolates have been identified based on microscopic examination of the spores that they produce, cultivation followed by analysis of the traits and distinctions of the colonies, and morphological characteristics such as conidial size,

texture, shape, and structure (Aizenberg et al., 2000; Portnoy et al., 2004; Shelton et al., 2002; Sterling and Lewis, 1998; Flappan et al., 1999). These methods are not only time consuming, potentially taking weeks for an identification to be made, but they are also highly inaccurate. It is often extremely difficult to distinguish different fungi based on morphological differences, and not all of the organisms present in an environmental sample will be culturable. These conditions allow for misidentification and understate the number of different organisms that constitute the microbial community. These conditions make it imperative to develop new methods of fungal identification that are rapid, highly sensitive, and specific.

To this end, numerous molecular techniques have been developed which are capable of identification and quantification of fungal species. Techniques such as quantitative Polymerase Chain Reaction (qPCR), restriction fragment length polymorphism (RFLP) analysis, random amplified polymorphic DNA (RAPD) analysis, and image analysis have all been used successfully to identify and quantify fungal organisms from a number of different environmental samples (Vesper et al., 1999; Velegraki et al., 1999; Isik et al., 2003; Dorge et al., 2000).

These methods enable rapid, sensitive, and specific identification of fungal organisms, however currently they are only being used to identify single organisms from complex environmental samples. Fungal organisms found in the environment are rarely if ever encountered singly. A more practical approach is the identification of numerous organisms from a single environmental sample. Identification of multiple fungal species in a single Polymerase Chain Reaction (PCR) can save time and money, while maintaining high specificity and accuracy. Here we describe a multiplex PCR method

that we have developed and optimized, which is capable of identifying four organisms by monitoring amplified DNA band migration following agarose gel electrophoresis.

2. Materials and Methods

2.1. *Fungal isolates*.

Aspergillus flavus ATCC# 11498, *A. fumigatus* ATCC# 1022 and *C. elatum* ATCC# 11280 were obtained from the American Type Culture Collection. *A. versicolor*, *A. parisiticus*, *A. proliferans*, *A. awamorii*, *A. sydowii*, *A. niger*, *P. glabrum*, *P. aurantiogriseum*, *P. chrysogenum*, *P. variabile*, *P. oxalicum*, *C. sphaerospermum*, *C. cladosporioides*, *Cladosporium* 3491, *Cladosporium* 3627, *Cladosporium* 3680, *S. chartarum*, *Stachybotrys* 3559, *Stachybotrys* 3657, and *Stachybotrys* 3514 were all kindly provided by Research Triangle Institute (RTI). Numbers following the organism name are our laboratory identification numbers. All of the organisms provided by RTI were environmental samples obtained from environmental dust samples from houses in Cleveland, Ohio. *Penicillium purpurogenum* was kindly provided by Steve Vesper from EPA/ORD/NERL. It was also isolated from environmental dust samples from houses in Cleveland, Ohio. The *Escherichia coli* DH5α was provided by Barbara Roop from EPA/ORD/NHEERL.

2.2. Growth and harvest of spores.

All fungal organisms were grown on Sabouraud Dextrose Agar (SDA) plates. Plates were prepared according to the suppliers instructions. Each organism was plated and grown to confluence on three different SDA plates in preparation for spore harvest. Organisms were allowed to grow for at least 10 days prior to spore harvest. Spores were

harvested as previously reported **(**Dean et al., 2004; Crow et al., 1994). 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 the three plates was combined and the spore suspension centrifuged at 12,000 X g for 5 min. The supernatant was then decanted leaving the spore pellet intact. The pellet was washed three times with 10 ml of phosphate buffer and stored at 4 °C until needed. The total spore counts were enumerated by direct microscopic counting on a hemacytometer as described by Roe et al. (2001).

2.3. Fungal DNA purification.

The spore DNA was purified as previously reported (Dean et al., 2004). The spores were mechanically broken open using a bead milling method followed by a phenol:$CHCl_3$ -ethanol precipitation step. For bead milling 0.25 g of acid-washed glass beads (212-311 μm) were placed in a 2 ml screw cap conical tube. 200 μl or approximately 10^7 spores were added to the glass beads. The tube was then shaken in a mini bead beater (Biospec Products, Bartlesville, OK) for 50 seconds at the maximal rate. The tube was then placed on ice for 1 minute to cool the sample and then shaken a second time. The supernatant was removed from the beads and subjected to a phenol:$CHCl_3$ extraction and an ethanol precipitation (Ausubel et al., 1994). Following precipitation the samples were stored at minus 20 °C until needed.

2.4. Primers and PCR conditions.

Oligonucleotides ranging in length from 16 to 24 mers were designed to amplify specific regions of *Aspergillus versicolor*, *Stachybotrys chartarum*, *Penicillium purpurogenum*, and *Cladosporium* spp. (Table 1). Positive control reactions were carried

out using forward primer ITS1 (5' TCCGTAGGTGAACCTGCGG 3') and reverse primer ITS4 (5' TCCTCCGCTTATTGATATGC 3') (White et al., 1990). These primers are considered universal fungal primers and have been shown to amplify the organisms used in this study (Wu et al., 2002). Initial PCR optimization consisted of obtaining amplification of each target gene under individual reaction conditions. Ultimately, all of the conditions for the multiplex reactions were the same except the concentrations of the primers used for each organism (Table 2). In the end, each PCR reaction contained: 0.2 mM each dNTP, 1.5 mM $MgCl_2$, 1.5 U Platinum 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 35 cycles of 96 °C 30 sec; 50 °C 15 sec; and 68 °C 2 min. PCR products were separated by electrophoresis in 2% low melting point agarose, and visualized by ethidium bromide staining.

3. Results and Discussion.

3.1. Rationale

In order to increase the efficiency and accuracy of fungal identification a multiplex PCR assay was developed which is capable of identifying *Aspergillus versicolor*, *Penicillium purpurogenum*, *Cladosporium* spp., and *Stachybotrys chartarum* based on the banding pattern of amplified genes following agarose gel electrophoresis. The above organisms were chosen based on studies which have shown them to be highly prevalent and possibly signature species that are found in unhealthy indoor environments (Vesper et al., 2004). The selected genes allow for specific amplification and species identification. The *Tri5* gene was targeted in *Stachybotrys chartarum* because it is the

first gene in the macrocyclic trichothecene biosynthetic pathway (Doohan et al., 1999). Therefore, in an environmental sample, amplification of the *Tri5* gene not only identifies *S. chartarum*, but also indicates the possibility of mycotoxin production.

3.2. PCR optimization and results

3.2.1 Individual organism PCR

Initial work focused on designing primers for the amplification of specific genes from each organism. A thorough search of NCBI followed by alignment and analysis with BioEdit software (Hall, 1999) allowed for the selection of primers capable of amplifying fragments that could be distinguished based on their size. Care was taken to ensure that primers would be compatible in a single reaction (no primer cross interactions) and that the melting temperature (Tm) for each was similar. Table 1 shows the primer sequences, the genes they amplify, their Tm, and the length of their individual reaction products.

Before multiplex PCR analysis, each organism was subjected to individual PCR to optimize the conditions necessary for sufficient amplification. The emphasis was on generating a positive result for all four organisms using the exact same reaction constituents and thermal cycler temperature profile. In multiplex PCR most of the constituents within the reaction must remain the same for each organism, however, amplification primers are constituents which can be unique for each organism within the multiplex reaction. In each case, a primer chessboarding scheme was used to determine the optimal concentration of both forward and reverse primers to reduce the presence of spurious amplification products (Gunson, et al., 2003). Figure 1 shows the removal of primer dimers and other unwanted bands from *Cladosporium cladosporioides* solely by

altering the concentrations of the primers within each reaction. The results for *Cladosporium* indicate that decreased concentrations of the forward primer greatly enhance the elimination of unwanted bands, while the reverse primer has a minimal effect on spurious band formation. The same primer chessboarding scheme was performed for each organism with optimal primer concentrations chosen based on the quality of the amplification product (data not shown) with the final primer reaction concentrations shown in Table 2. Once each reaction had been optimized, 5% of each PCR was run on a 2% agarose gel and analyzed for the ability to separate the products based on their size (figure 2). Figure 2 clearly shows the purity of the amplified bands and the ability to distinguish each organism based on the size of the resulting PCR product.

3.2.2 Multiplex PCR

Ultimately the multiplex PCR reactions were set up according to Table 3. In order to achieve maximal amplification of each gene within the multiplex reaction different reagent concentrations were used (Henegariu et al., 1997; Markoulatos et al., 2002). In all cases the *Stachybotrys chartarum Tri5* gene was the most difficult to amplify. It was decided that a possible reason could be because of the low copy number of the *Tri5* gene, while both *Penicillium* and *Cladosporium* reactions utilized the higher copy number ribosomal DNA genes. This could result in reagents and polymerase being preferentially used to amplify the ribosomal genes.

To counter this affect, we ran multiplex reactions with a 2 X concentration of *Taq* buffer. Increasing the buffer concentration had no affect on the amplification of the target genes, however, it did cause unwanted reaction products to develop making identification of the desired bands very difficult (data not shown).

Another reaction constituent that was modified was the concentration of $MgCl_2$. The final reaction $MgCl_2$ concentration was 1.5 mM. We attempted up to 2.5 mM final $MgCl_2$ concentrations in an effort to increase the intensity of the amplified product bands. The only consequence of increasing the $MgCl_2$ was an increase in the formation of unwanted bands (data not shown).

Finally, an attempt was made to increase the concentration of *Taq* polymerase within the multiplex reaction. Polymerase concentrations were raised up to 2 units per reaction. Increasing the concentration of the polymerase did increase the intensity of the target bands, however, once again numerous other spurious products became visible which confounded the analysis (data not shown).

Final reaction conditions can be seen in tables 2 and 3 with the results in figure 3. Using these conditions, the results of a multiplex reaction containing 10^4 spore equivalents of *Cladosporium*, 10^5 spore equivalents of *Stachybotrys*, and 10^4 spore equivalents *Aspergillus* were favorable with each target showing excellent amplification. When the fourth organism (10^4 spore equivalents of *Penicillium*) was added to the reaction all of targets amplified well. One slight exception to this was the amplification of *Cladosporium* which decreased in intensity with the addition of *Penicillium*. However, all of the organisms amplified sufficiently with no spurious band formation enabling the identification of each organism based on individual band migration.

To ensure that the multiplex reaction was specific for the target organisms each primer pair was run in reactions with other genomic DNA. These results can be seen in Table 4, whereby it is shown that all of the primers are specific for their target organism. Table 4 also shows the usefulness of using the *Tri5* gene as a target for *Stachybotrys*

chartarum. Each *S. chartarum* isolate tested generated a positive band for the *Tri5* gene, which could be used to alert building occupants and remediators to the potential presence of macrocyclic trichothecene production.

4. Conclusions

Due to the inherent difficulties and inaccuracies associated with attempting to distinguish fungal organisms based on growth and morphological characteristics our laboratory set out to develop and optimize a multiplex PCR reaction capable of identifying four fungal species. We accomplished this when we were able to identify *Stachybotrys chartarum*, *Penicillium purpurogenum*, *Aspergillus versicolor*, and *Cladosporium* spp. based on the mobility of their amplified genes during agarose gel electrophoresis. These results are a first step which clearly shows the potential of using multiplex PCR in the future for the identification of environmental fungal isolates.

Future work extending on the above results includes development of a multiplex reaction that is quantitative for four to five organisms. This will enable not only the identification of each organism, but will show the numbers of each organism present in the environmental sample. The generation of the above multiplex reaction is the first step in multiple organism identification, and shows the usefulness of multiplex PCR in the analysis of environmental fungal samples.

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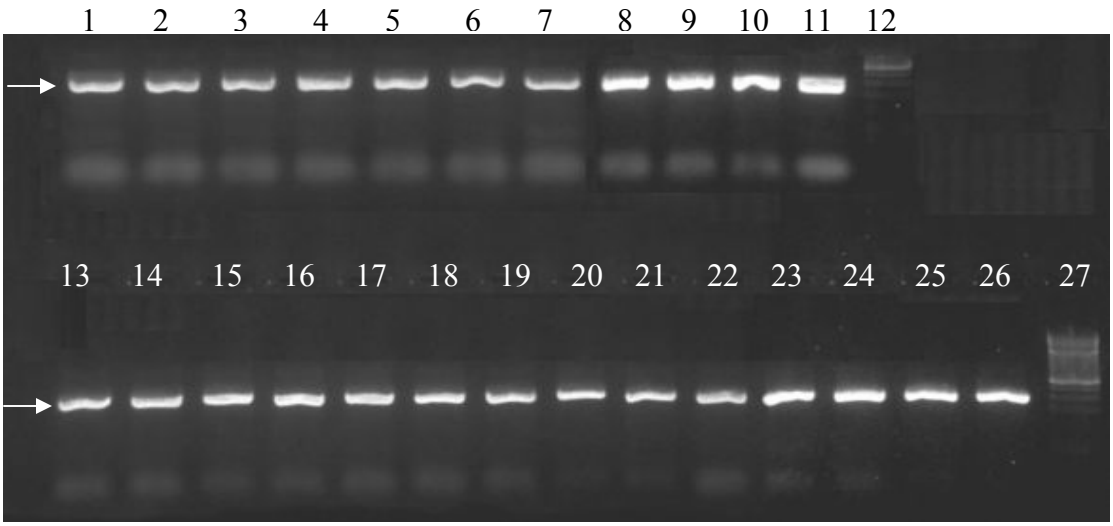

Lane	1	2	3	4	5	6	7	8	9	10	11	12	13
Forward	5μM	5μM	5μM	5μM	5μM	4μM	4μM	4μM	4μM	4μM	3μM	---	3μM
Reverse	5μM	4μM	3μM	2μM	1μM	5μM	4μM	3μM	2μM	1μM	5μM	---	4μM
Lane	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**
Forward	3μM	3μM	3μM	2μM	2μM	2μM	2μM	2μM	1μM	1μM	1μM	1μM	1μM
Reverse	3μM	2μM	1μM	5μM	4μM	3μM	2μM	1μM	5μM	4μM	3μM	2μM	1μM

Figure 1. Results showing primer concentrations in primer chessboarding method of PCR optimization for *Cladosporium* spp. Optimal results are at low concentrations of forward primer. White arrows indicate 410 bp product.

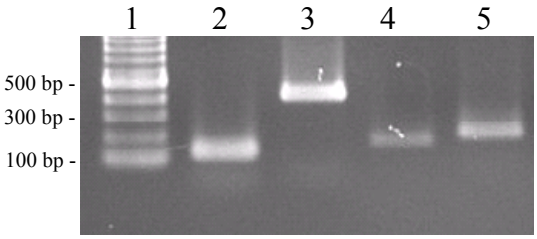

Figure 2. Results showing individual amplification of indoor fungal contaminants. Lane one shows the size marker with sizes (bp) on the left. Lane two contains the amplified β-Tubulin fragment from *A. versicolor*, lane three the amplified ribosomal fragment from *Cladosporium*, lane four the amplified Tri5 fragment from *S. chartarum*, and lane five the amplified ribosomal fragment from *P. purpurogenum*. Individual reactions clearly show the purity of the reaction products and their distinct sizes for identification purposes.

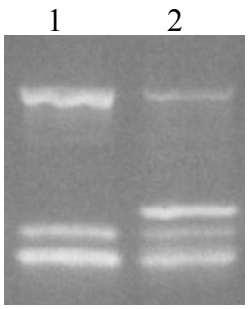

Figure 3. Results showing multiplex reactions. Lane one contains *Cladosporium* (top band, 410 bp), *S. chartarum* (middle band, 165 bp), and *A. versicolor* (lower band, 127 bp). Lane two shows the addition of *P. purpurogenum* (195 bp). Band intensity and separation allow for identification of the four species.

Table 1
Sequence and data for primer pairs used in this study

Target Organism	Target gene	Primer	Nucleotide Sequence (5' → 3')	Tm °C	Product length
S. chartarum	**Tri5**	ST5F	GTGGCAACCCGCAAAAGC	58	165 bp
		ST5R	TTGCTCTTTCTTGGAATATTTTGG	54	
P.Purpurogenum	ITS1 to 5.8S rDNA	PurF	ACCCTAATGAAGAAGGACTGTCTG	57	195 bp
		PurR	CGCTCTCGGACAGGCA	57	
A. versicolor	β-Tubulin	AspBTF	CATCCATTTCAGATGGTATTTCCT	54	127 bp
		AspBTR	TGTTTTGATCGAGTCTTGGACG	57	
Clad. spp.	ITS1 to 28S rDNA	CladF	CCKGGATGTTCATAACCCTTTG	56	410 bp
		CladR	CCCGAACACCCTTTAGCG	56	

Table 2
Final optimized primer concentrations
for multiplex PCR reactions

Organism	Forward	Reverse
Cladosporium spp.	1 μM	1 μM
A. versicolor	1 μM	1 μM
S. chartarum	125 nM	1 μM
P. purpurogenum	125 nM	250 nM

Table 3
Reaction conditions used in PCR optimization assays.

Reagent	Initial Concentration	Volume	Final Concentration
10 dNTPs	10 mM	1.0 μl	0.4 mM
10X *Taq* Buffer	10 X	2.5 μl	1 X
Forward Primer	25 μM	1.0 μl	1 μM
Reverse Primer	25 μM	1.0 μl	1 μM
Template	Variable	2.5 μl	Variable
$MgCl_2$	25 mM	1.5 μl	1.5 mM
dH_2O	--	15.35 μl	--
Taq Polymerase	5U/μl	0.15 μl	0.75 U
Final Volume		**25.0 μl**	

Table 4

Organism	*A. versicolor* primer pair	*Cladosporium* primer pair	*S. chartarum* primer pair	*P. purpurogenum* primer pair
Aspergillus versicolor	+	-	-	-
Aspergillus flavus	-	-	-	-
Aspergillus fumigatus	-	-	-	-
Aspergillus parisiticus	-	-	-	-
Aspergillus proliferans	-	-	-	-
Aspergillus awamorii	-	-	-	-
Aspergillus sydowii	-	-	-	-
Aspergillus niger	-	-	-	-
Penicillium purpurogenum	-	-	-	+
Penicillium glabrum	-	-	-	-
Penicillium aurantiogriseum	-	-	-	-
Penicillium chrysogenum	-	-	-	-
Penicillium variabile	-	-	-	-
Penicillium oxalicum	-	-	-	-
Clad. sphaerospermum	-	+	-	-
Clad. cladosporioides	-	+	-	-
Clad. elatum	-	+	-	-
Clad. 3491	-	+	-	-
Clad. 3627	-	+	-	-
Clad. 3680	-	+	-	-
Stachybotrys chartarum	-	-	+	-
S. chartarum 3559	-	-	+	-
S. chartarum 3657	-	-	+	-
S. chartarum 3514	-	-	+	-
Escherichia coli DH5α	-	-	-	-

Negative controls and specificity of multiplex primer pairs