

Development and evaluation of a real-time quantitative PCR assay for *Aspergillus flavus*

Patricia Cruz¹
Mark P. Buttner

Microbiology Division, Harry Reid Center for
Environmental Studies, University of Nevada,
Las Vegas, 4505 S. Maryland Parkway, Box 4009,
Las Vegas, Nevada 89154-4009

Key words: analytical/rapid methods, detection,
Fungi, identification, PCR

INTRODUCTION

Abstract: *Aspergillus flavus* is a ubiquitous mold and the most common mold contaminating foodstuffs. Many strains of *A. flavus* produce aflatoxins. In addition it is an allergen and an opportunistic pathogen of animals and plants. *A. flavus* often is underestimated in traditional culture analyses due to the expertise required and the cost associated with speciating members of the genus *Aspergillus*. The goal of this study was to develop and validate a primer and probe set for the rapid detection and quantitation of *A. flavus* in pure culture using real-time quantitative polymerase chain reaction (QPCR) amplification. Unique DNA regions were located in the genome of the target organism by sequence comparison with the GenBank database, and several candidate oligonucleotides were identified from the scientific literature for potential use with the TaqMan[®] QPCR technology. Three primer and probe sets were designed and validated for specificity and sensitivity in laboratory experiments. Initial screening to test for sensitivity was performed with seven *A. flavus* isolates and selected nontarget fungi. Specificity testing was conducted with the selected primer and probe set, which amplified all nine *A. flavus* isolates tested, including an aflatoxin producing strain. The primers did not amplify DNA extracted from 39 other fungal species (comprising 16 genera), including 18 other *Aspergillus* species and six *Penicillium* species. No amplification of human or bacterial DNA was observed; however cross-reactivity was observed with *Aspergillus oryzae*. PCR analysis of DNA dilutions and the use of an internal positive control demonstrated that 67% of the fungal DNA samples assayed contained PCR inhibitors. The assay validated for the target organism is capable of producing PCR results in less than 1 h after DNA extraction. The results of this research demonstrate the capabilities of QPCR for the enhanced detection and enumeration of fungi of significance to human health.

Aspergillus flavus is a ubiquitous mold, one of the most common of the *Aspergillus* species and is the most widely reported fungus contaminating foodstuffs (Kwon-Chung and Bennett 1992, Klich 2002). It is commonly found in (ground) nuts, cereals, oil seeds and spices and has been found occasionally in dried fruit (Samson et al 2000). *A. flavus* is the main producer of aflatoxins, with strains that produce aflatoxin B₁, the most potent liver carcinogen known, and aflatoxin B₂ (Pitt and Hocking 1999). Worldwide, aflatoxins are the most important mycotoxins in foodstuffs and they can produce acute and chronic toxicity in animals and humans (Pitt and Hocking 1999). The U.S. Food and Drug Administration has established total aflatoxin quantities allowed in food or feed. In addition the high carcinogenicity produced by this mycotoxin in animals justifies every effort to monitor and reduce it in foods (Pitt and Hocking 1999). It also has been reported that spores of *A. flavus* can contain a high concentration of aflatoxins, up to 1000 µg/g; therefore monitoring and removal of this mold also is warranted for fungal contaminated indoor environments (Pitt and Hocking 1999).

In addition to its importance as a contaminant of certain agricultural commodities *A. flavus* is an opportunistic pathogen that can cause pulmonary mycosis in immunosuppressed individuals after inhalation of aerosolized spores (Cole and Samson 1984). Of the hundreds of *Aspergillus* species *A. flavus* is one of several that has been reported routinely as the causative agent of human infections. Accurate measurement of *A. flavus* spores in environmental samples is important; however it often is underestimated in traditional culture analyses due to the expertise required in fungal identification and the cost associated with speciation of members of genus *Aspergillus*.

The research conducted in this study used the polymerase chain reaction (PCR), which detects specific microorganisms by amplifying DNA sequences unique to the organism of interest and does not depend on the culturability of the target organism. To use PCR, sequence information must be identified

for a specific target DNA region. Once a unique DNA segment has been identified oligonucleotide primers are designed, synthesized and tested for sensitivity, specificity and selectivity. A fluorogenic nuclease assay recently was developed as a means to rapidly amplify and quantitate PCR products, eliminating the need for post-PCR manipulations (Heid et al 1996). This assay uses a fluorogenic probe that anneals between the primers of choice as the amplification reaction proceeds, allowing for the determination of starting copy number of target DNA. The 5' nuclease assay that is integral to this technique has been validated for the detection of microorganisms, including *Salmonella* (Nogva and Lillehaug 1999), *Mycobacterium tuberculosis* (Desjardin et al 1998) and *Stachybotrys chartarum* (Cruz-Perez et al 2001b).

Several investigators have published methods for the detection of *A. flavus* in different matrices using conventional PCR followed by agarose gel electrophoresis (Shapira et al 1996, Einsele et al 1997, Zhou et al 2000), PCR and enzyme immunoassay (de Aguirre et al 2004, Hinrikson et al 2005a) and multiplex PCR assays with biprobes (Schabereiter-Gurtner et al 2007). Although faster and more sensitive than culture analysis for detection, some of these methods require post-PCR manipulations and are only semiquantitative. Einsele et al (1997) designed universal primers and several hybridization probes for the detection of fungi in blood specimens. However the group-specific *Aspergillus* probe and the *A. fumigatus*/*A. flavus*/*A. versicolor* probe cross-reacted with other species. Other researchers have employed PCR for the amplification of a large DNA segment followed by DNA sequencing to identify *Aspergillus* species by comparison to sequence data in genetic repositories such as GenBank (Henry et al 2000, Hinrikson et al 2005b).

More recently researchers have used PCR with universal primers followed by hybridization to an oligonucleotide probe or microarray for the detection of fungal pathogens (Martin et al 2000, Leinberger et al 2005, Huang et al 2006). While the microarray approach can discriminate simultaneously among fungal targets, some of these assays require further validation, employ post-PCR manipulations or are only semiquantitative. The goal of this study was to develop oligonucleotide primers and a probe for the rapid detection and absolute quantitation of *A. flavus* in pure culture with real-time quantitative PCR. This assay can be applied to the analysis of field samples and is expected to have use in indoor air quality monitoring and quality control of agricultural commodities and feed.

MATERIALS AND METHODS

Test organisms and culture media.—*Aspergillus flavus* served as the target organism for this study. Forty-eight fungal isolates, representing 16 genera, were obtained from the American Type Culture Collection (ATCC, Manassas, Virginia), the National Center for Agricultural Utilization Research, U.S. Department of Agriculture (formerly the Agricultural Research Service Culture Collection of the Northern Regional Research Laboratory) or from laboratory stocks maintained at the Harry Reid Center for Environmental Studies, University of Nevada, Las Vegas (TABLE I). *Eurotium herbariorum* and *Aspergillus restrictus* isolates were grown on Czapek yeast agar with 20% sucrose (Klich 2002), all other isolates were cultured with malt extract agar, MEA (Klich 2002). Pure cultures of the test organisms were subcultured with a sterile swab that was streaked over the entire agar surface. All fungi were incubated at 25 C and examined periodically with a stereomicroscope until substantial sporulation was observed (3–20 d).

Primer selection and design.—*A. flavus* sequences for the 18S, 5.8S, and 28S rRNA genes were obtained from GenBank (National Center for Biotechnology Information, National Institutes of Health) and compared against all other sequences available online with the basic local alignment search tool algorithm (BLAST, National Center for Biotechnology Information) (Altschul et al 1990). In addition several candidate oligonucleotides were identified from the scientific literature for potential use with TaqMan® QPCR technology (White et al 1990, Leinberger et al 2005, Huang et al 2006). The melting temperature (T_m) and percent Guanine-Cytosine (GC) of these candidate primers and probes were determined with the Primer Express software package (Applied Biosystems, Foster City, California), and their location was identified in existing *A. flavus* sequences (i.e. GenBank accession Nos. DQ683119 and EF121332). Three primer and probe sets were created from the candidate oligonucleotides, and their sequences were elongated or shortened to obtain melting temperatures conforming to the Primer Express design criteria. Amplicon sizes were 212–249 bp. An *Aspergillus* group-specific probe (Asp1S) designed by Leinberger et al (2005) was used without modification as the forward primer for all three primer and probe sets designed (TABLE II). Likewise the same fluorescent probe was used for all three primer and probe sets. Probe No. 11, designed by Huang et al (2006) for the microarray hybridization of *A. flavus*, was modified by deleting the first four bases on the 5' end and the last six bases on the 3' end and renamed AfP (TABLE II). Three reverse primers were tested, primer ITS4 (White et al 1990) without modifications, AfIR2 and AfIR3. The last 18 bases in the 3' end of the universal fungal probe No. 2 (Huang et al 2006) were deleted to create AfIR2; for AfIR3 deletions consisted of the first 17 bases in the 5' end and the last base on the 3' end. The resulting primer and probe sequences are provided (TABLE II). Primers were obtained from Operon Biotechnologies (Huntsville, Alabama), resuspended with nuclease-free water (Promega Corp., Madison, Wisconsin) and stored at –70 C. The fluorescent probe was

TABLE I. Test microorganisms and PCR results

Species name	Accession number	PCR results	
		<i>A. flavus</i>	Inhibition
<i>Acremonium strictum</i>	ATCC 10141	—	—
<i>Aspergillus caespitosus</i>	NRRL 1929 ^a (ATCC 11256)	—	+
<i>Aspergillus candidus</i>	NRRL 303 ^a (ATCC 1002)	—	+
<i>Aspergillus carneus</i>	NRRL 527 ^a (ATCC 16798)	—	—
<i>Aspergillus clavatus</i>	HRC	—	+
<i>Aspergillus flavipes</i>	HRC	—	—
<i>Aspergillus flavus</i>	HRC	+	+
<i>Aspergillus flavus</i>	NRRL 453 ^a (ATCC 11500)	+	+
<i>Aspergillus flavus</i>	NRRL 485 ^a (ATCC 11492)	+	+
<i>Aspergillus flavus</i>	NRRL 492 (ATCC 11495)	+	+
<i>Aspergillus flavus</i>	NRRL 500 ^b (ATCC 16875)	+	+
<i>Aspergillus flavus</i>	NRRL 501 (ATCC 9170)	+	+
<i>Aspergillus flavus</i>	NRRL 3537 (ATCC 9643)	+	+
<i>Aspergillus flavus</i>	NRRL 3646 (ATCC 11655)	+	+
<i>Aspergillus flavus</i>	NRRL 4818 ^a (ATCC 16870)	+	+
<i>Aspergillus fumigatus</i>	ATCC 36607	—	+
<i>Aspergillus japonicus</i>	HRC	—	+
<i>Aspergillus niger</i>	ATCC 10535	—	+
<i>Aspergillus ochraceus</i>	HRC	—	+
<i>Aspergillus oryzae</i>	NRRL 5590 (ATCC 42149)	+	+
<i>Aspergillus parasiticus</i>	ATCC 15517 ^{a b}	—	+
<i>Aspergillus restrictus</i>	NRRL 145	—	—
<i>Aspergillus sydowii</i>	HRC	—	+
<i>Aspergillus tamari</i>	NRRL 20818 ^a	—	+
<i>Aspergillus terreus</i>	HRC	—	+
<i>Aspergillus unguis</i>	HRC	—	+
<i>Aspergillus ustus</i>	HRC	—	—
<i>Aspergillus versicolor</i>	NRRL 238 ^a (ATCC 9577)	—	+
<i>Aspergillus wentii</i>	NRRL 26310	—	—
<i>Aureobasidium pullulans</i>	ATCC 16622	—	—
<i>Bipolaris</i> sp.	HRC	—	—
<i>Chaetomium</i> sp.	HRC	—	—
<i>Cladosporium herbarum</i>	ATCC 28987	—	—
<i>Emicella nidulans</i>	NRRL 189 (ATCC 16855)	—	+
<i>Eupenicillium hirayamae</i>	NRRL 3588	—	+
<i>Eurotium herbariorum</i>	NRRL 116 ^a (ATCC 16469)	—	—
<i>Neosartorya quadricincta</i>	NRRL 2221	—	+
<i>Paecilomyces variotii</i>	ATCC 22319	—	+
<i>Penicillium brevicompactum</i>	HRC	—	+
<i>Penicillium chrysogenum</i>	ATCC 9480	—	+
<i>Penicillium commune</i>	HRC	—	+
<i>Penicillium crustosum</i>	NRRL 968 (ATCC 10430)	—	+
<i>Penicillium purpurogenum</i>	HRC	—	—
<i>Penicillium simplicissimum</i>	NRRL 1075 (ATCC 10482)	—	—
<i>Phoma</i> sp.	HRC	—	—
<i>Stachybotrys chartarum</i>	ATCC MYA-3310	—	+
<i>Talaromyces flavus</i>	HRC	—	—
<i>Trichoderma</i> sp.	HRC	—	—
Human DNA, 15 ng	ABI	—	—
<i>Bacillus cereus</i> , 10 ng	ATCC 14579D ^a	—	—

Test microorganisms and PCR results obtained for the specificity testing of the *A. flavus* primers and probe Asp1S, AflR2 and AflP, respectively. Inhibition of the PCR assay was determined with an internal positive control spiked into each sample (+ indicates amplification; — indicates no amplification; + and — in the Inhibition column refer to the presence or absence of PCR inhibitors, respectively; NRRL = Northern Regional Research Laboratory, U.S. Department of Agriculture; HRC = Harry Reid Center for Environmental Studies, University of Nevada at Las Vegas; ATCC = American Type Culture Collection; ABI = Applied Biosystems, Foster City, California).

^a Type strain

^b Aflatoxin-producing strain

TABLE II. Primers and probes tested

Oligonucleotide	Name	Sequence (5'→3')	Target	Reference
Forward primer	Asp1S	ATGCCTGTCCGAGCGT	5.8S rRNA	(Leinberger et al 2005)
	ITS4	TCCTCCGCTTATTGATATGC	28S rRNA	(White et al 1990)
Reverse primer	AflR2	TTAAGTTCAGCGGGTATRCC	28S rRNA	(Huang et al 2006)
	AflR3	RCCTACCTGATRRGAGGTCA	28S rRNA	(Huang et al 2006)
Probe	AflP	6-FAM-CGCTTGCCGAACGCAAATCAATCTT-TAMRA	ITS2	(Huang et al 2006)

obtained from Applied Biosystems, diluted with Tris-EDTA buffer (TE, pH 8.0) and stored at -70°C . All three primer sets initially were tested for selectivity with several *A. flavus* isolates and selected nontarget fungi. After this screening the most selective and sensitive primer and probe set was selected for testing with all organisms (TABLE I).

Specificity testing.—For PCR primer specificity testing pure cultures of the test organisms (TABLE I) were grown as indicated above and sampled by gently rolling a cotton swab over the surface of the fungal mat. The swab was rolled until it was coated with spores and resuspended in a 15 mL conical centrifuge tube containing 3 mL 0.01 M potassium phosphate buffer with 0.05% Tween 20 (Sigma Chemical Co., St Louis, Missouri) (PBT, pH 7.0). After vortexing on maximum speed 1 min the swab was removed aseptically and the spore suspension stored at -70°C for DNA extraction. Extraction was performed as described below. Nontarget DNA (TABLE I) was subjected to PCR amplification with the selected primer and probe set for *A. flavus*. A universal fungal PCR assay developed in our laboratory was used to confirm the presence of DNA in all extracts from test organisms assayed with the selected *A. flavus* primer and probe set (data not shown).

DNA extraction.—*Bacillus cereus* DNA (ATCC 14579D) was diluted with TE buffer to a concentration of 2 ng/ μL . Human DNA at a concentration of 3 ng/ μL was obtained from Applied Biosystems. All fungal DNA was extracted with the alternative protocol of the Ultra Clean DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, California) with these modifications: 200 μL of the liquid spore harvest was extracted, and the DNA elution buffer consisted of 50 μL TE buffer. DNA was stored at -70°C .

PCR amplification.—The 7900HT Fast Real-Time PCR System (Applied Biosystems) was used for PCR analysis. Optimized conditions recommended by the manufacturer were used, and the effect of bovine serum albumin (BSA) as a PCR enhancer was investigated. Amplification conditions with the Applied Biosystems reagents (with final concentrations shown) were: fungal DNA template (5 μL); 1 $\times$ TaqMan[®] Fast Universal PCR Master Mix, No AmpErase[®] UNG; forward and reverse primers, 0.9 μM each primer; and 0.2 μM probe. The reaction volume was adjusted to 25 μL with nuclease-free water. Cycling conditions in Fast mode consisted of 95 $^{\circ}\text{C}$ for 20 s, and 40 cycles at 95 $^{\circ}\text{C}$ for 1 s followed by 60 $^{\circ}\text{C}$ for 20 s. All samples and standards were amplified in duplicate, and negative and positive controls were included with each PCR assay. After amplification the data were analyzed and plotted (fluorescence vs. cycle

number) using the software provided with the PCR instrument. The extent of amplification was reported by the software as the mean C_T value of two replicates. C_T refers to the PCR cycle number where detectable amplification product is measured and is inversely proportional to the initial DNA template concentration.

PCR quantitation standards.—*A. flavus* quantitation standards of known concentration were prepared to determine the lower detection limit (LDL) of the assay with protocols developed in our laboratory, with modifications (Cruz-Perez et al 2001b, a). Briefly, dry spores were obtained by three-point inoculation of 10 MEA Petri dishes with *A. flavus* NRRL 4818 and incubation at 25 $^{\circ}\text{C}$ for 14 d. The dishes were inverted onto a sterile glass funnel and gently tapped to dislodge spores. Several loopsfull of dry *A. flavus* spores were resuspended in 10 mL PBT and vortexed at maximum speed for 1 min. The total concentration of the *A. flavus* NRRL 4818 spore suspension was determined with a Multisizer[™] 3 electronic particle counter (Beckman Coulter Inc., Miami, Florida). An aliquot of the spore suspension was diluted in filtered Isoton II solution (Beckman Coulter Inc.) and enumerated with the electronic particle counter. The particles corresponding to the spore peak were counted and the data were adjusted automatically for coincidence correction by the instrument. The data from 10–50 μL aliquots of the sample were averaged and the concentration of total spores per mL in the spore suspension was determined. Aliquots of the enumerated spore suspension (10^1 to 10^6 spores/PCR reaction) were diluted serially in PBT and stored at 4 $^{\circ}\text{C}$ overnight for DNA extraction. Standards were prepared with the same extraction and purification methods used to process fungal DNA samples. Replicate standards (10^{-1} – 10^5 *A. flavus* DNA templates or spore equivalents/reaction) were amplified with the same conditions employed for samples. PCR amplification efficiency (E) was estimated from the slope of the standard curve with the formula $E = (10^{-1/\text{slope}}) - 1$. At 100% efficiency the slope equals -3.32 . The goodness of fit of the linear regression correlation coefficient (r^2) and slope were used to assess the quality of the PCR standards.

Determination of PCR inhibitors.—An internal positive control (IPC-Vic Probe, Applied Biosystems) was incorporated into the PCR reaction to determine the presence of PCR inhibitors. The IPC kit contains control IPC DNA (1 $\times$ final concentration) and an IPC Mix (1 $\times$ final concentration) of oligonucleotide primers and a fluorescent probe. The dye used is different from that of the target DNA probe to allow for the differentiation of fluorescent signals

generated during amplification. DNA from all test microorganisms was assayed for the presence of PCR inhibitors. DNA from selected samples was assayed undiluted or diluted 10- or 100-fold.

RESULTS

Primer selection and design.—BLAST analyses on the internal transcribed spacer regions (i.e. ITS1 and ITS2) of the *A. flavus* genome revealed a high degree of sequence homology with *Aspergillus* spp. and genus *Penicillium*. Of the greater than 1700 matches obtained for these *A. flavus* sequences, 52% belonged to *Aspergillus* spp. and 22% to genus *Penicillium*. When homology searches were conducted for the ITS1 and ITS2 regions separately approximately 25% of the matches belonged to genus *Aspergillus* and approximately 60% to genus *Penicillium*.

Initial screening experiments with the three primer sets were conducted with seven *A. flavus* isolates. PCR results showed that the Asp1S/AflR2 primer set had better sensitivity (C_T values were 18.78–30.51) than the Asp1S/ITS4 (C_T values = 20.44–40.00) and the Asp1S/AflR3 (C_T values = 25.75–33.48) primer sets. Two of the *A. flavus* isolates (NRRL 492 and NRRL 3646) produced erratic amplification plots with the Asp1S/ITS4 primer set. The NRRL 492 strain also produced erratic plots with the Asp1S/AflR2 primer set. The only negative PCR result during these screening experiments was seen with the Asp1S/ITS4 primer set. Due to the poor sensitivity seen with this set, the Asp1S/ITS4 was rejected as a candidate primer set. Additional screening with selected nontarget organisms was performed to determine cross-reactivity but only with the other two primer sets. Equivalent PCR results were obtained for the Asp1S/AflR2 and Asp1S/AflR3 primer sets during this secondary screening; therefore the Asp1S/AflR2 primer set was selected for specificity testing based on sensitivity results obtained with the *A. flavus* isolates.

Specificity testing.—Specificity testing results obtained with the selected primer and probe set are provided (TABLE I). The *A. flavus* primer and probe set amplified all nine *A. flavus* isolates tested. The primers did not amplify DNA extracted from 39 other fungal species (comprising 16 genera), including 18 non-*flavus Aspergillus* species and six *Penicillium* species. No amplification was observed of the human or bacterial DNA; however cross-reactivity was observed with *Aspergillus oryzae*.

PCR quantitation standards.—*A. flavus* PCR quantitation standards produced a linear standard curve with a correlation coefficient (r^2) of 0.995 and a slope

of -3.48 . The assay had a linear range of 10^{-1} – 10^3 DNA templates per PCR sample and an efficiency of 94%. IPC results showed that inhibition of the PCR assay was observed in the 10^4 and 10^5 standards. The lowest dilution for which a positive result was obtained 100% of the time was 10^{-1} ($n = 10$), corresponding to 0.4 DNA templates per PCR reaction or 80 DNA templates per mL of sample.

Determination of PCR inhibitors.—An internal positive control produced a baseline C_T value of approximately 27 when amplified with nuclease-free water (i.e. no template control), but a higher C_T value was observed when PCR inhibitors were present. Thirty-two of the 48 fungal DNA samples assayed contained PCR inhibitors, including all *A. flavus* isolates tested (TABLE I). Positive results obtained after serial 10-fold dilutions further demonstrated the presence of PCR inhibitors (data not shown). No PCR inhibitors were observed in the human or bacterial DNA samples.

DISCUSSION

Molecular biological techniques have been used effectively for the enhanced detection of microorganisms, overcoming the limitations associated with traditional culture analysis and producing fast, sensitive results. The pathogenicity of *A. flavus* and the ability of certain isolates to produce potent aflatoxins warrant the development of a fast, reproducible and quantitative assay capable of detecting this organism in agricultural commodities, livestock feed and indoor environments. However PCR-based methods currently available for the detection of *A. flavus* employ conventional PCR and gel electrophoresis, hybridization probes or DNA sequencing followed by comparison to sequence data in genetic repositories. Multiple DNA manipulations can result in cross-contamination, delays in the reporting of results and assays that are semiquantitative at best. Melting temperature profiles have been used for the detection of *A. flavus* and other fungal species; however the use of biprobes does not allow for quantitation of the target organism (Schabereiter-Gurtner et al 2007) and SYBR® green dye will bind and detect all double-stranded DNA, including nonspecific reaction products (Bu et al 2005). Furthermore several of the existing PCR assays have been validated only partially or not validated at all for cross-reactivity with DNA obtained from nontarget organisms. Niessen recently published a scientific literature review of PCR-based assays developed between 1996 and 2006 for the detection of mycotoxin producing fungi. He observed that the amount of species included in these studies for the assessment

of cross-reactivity varied considerably and concluded that primer sets should be tested for cross-reactivity with at least those species that are most closely related to the target organism (Niessen 2007). Various PCR assays have targeted genes involved in aflatoxin biosynthesis (Geisen 1996, Shapira et al 1996, Mayer et al 2003). However because mycotoxin biosynthesis is a complex process that can be influenced highly by environmental factors, finding a correlation between the PCR amplification signal and the concentration of the target mycotoxin(s) seems unlikely until assays based on gene expression are developed (Niessen 2007).

For the identification of a unique *A. flavus* target region for the development of specific PCR primers emphasis was placed on searching sequences of the 18S rRNA gene, which contains both highly variable and highly conserved regions (White et al 1990). Sequence comparison results on the internal transcribed spacer regions of the *A. flavus* genome revealed a high degree of homology with *Aspergillus* and *Penicillium* species. While members of genus *Aspergillus* are closely related to *Penicillium* species it is likely that the higher degree of sequence homology seen with *Penicillium* spp. was due to the limited amount of sequence information available for many *Aspergillus* species. This phenomenon has been reported by other researchers. Although Hinrikson et al (2005b) indicate that the GenBank database is lacking ITS sequences for some species, they demonstrated that these same regions are more reliable than other regions for the identification of medically important *Aspergillus* species. While Henry et al (2000) indicate that both ITS1 and ITS2 were necessary to accurately identify *Aspergillus* species using conventional PCR, more recently Huang et al (2006) observed interspecies variability in the ITS2 region and exploited this variation to design microarray probes for the identification of pathogenic fungi.

Oligonucleotides published in the scientific literature targeting the ITS2 and the 28S rRNA gene were modified as needed to conform to the optimal primer design criteria amenable with the assay and instrumentation used in the current study. While modifications were unnecessary for the forward primer, the *Aspergillus* group-specific probe designed by Leinberger et al (2005), modifications were needed for the reverse primer and probe. The results of the present study showed that targeting the ITS2 region and a small portion of the 28S rRNA gene specifically detected *A. flavus*. The protocol has a sensitivity of <1 DNA template or spore equivalent per PCR reaction and allows for a much faster detection and quantitation of *A. flavus* from pure culture compared

with culture analysis, which might take several days. The use of known concentration standards prepared with the same extraction and purification methods used to process fungal DNA samples allows absolute quantitation of *A. flavus* DNA templates in samples. The high sensitivity of detection was largely a result of amplifying a gene found in multiple copy numbers, an advantage that has been observed by other researchers (Sandhu et al 1995, Henry et al 2000, Leinberger et al 2005).

Cross-reactivity testing is important for any primer and probe set to exclude false positive results that could arise from a lack of specificity in the assay system (Wu et al 2002). Although a DNA sequence comparison with genetic repositories may show that the selected primer set will amplify only the target organism it is imperative that the primer and probe set be challenged in the laboratory with DNA extracted from several nontarget organisms to confirm that they will not cross-react with nontarget DNA (Cruz and Buttner 2005, Niessen 2007). The DNA from each challenge microorganism is subjected to QPCR amplification with the primers and probe designed for the target organism, and if the primer and probe set is selective DNA from nontarget organisms will not be amplified. Appropriate controls must be included with these tests to corroborate that a negative QPCR result is due to the absence of cross-reactivity and not to the absence of DNA or the presence of PCR inhibitors. In this study the *A. flavus* primer and probe set successfully amplified all the target isolates tested and did not amplify DNA extracted from 39 nontarget fungal species. However cross-reactivity was observed with *A. oryzae*, a closely related species of *A. flavus*. Other researchers using PCR-based assays have indicated that *A. oryzae* is essentially identical to *A. flavus* (Geisen 1996). It is believed that *A. oryzae* is a domesticated form of *A. flavus*, and although it was reduced to *A. flavus* var. *oryzae* this taxon change has not been accepted due to the importance of *A. oryzae* in the production of fermented foods (Pitt and Hocking 1999, Blumenthal 2004). It has been reported that *A. oryzae* is rarely found in sources other than fermented foods (Pitt and Hocking 1999).

Research has shown the presence of PCR inhibitors in the fungal species *Aspergillus fumigatus*, *Alternaria chlamydospora* and *Stachybotrys chartarum* (Zhou et al 2000; Cruz-Perez et al 2001a, 2001b). Inhibitors commonly act by degrading or binding to the target DNA, rendering it nonavailable for amplification or by affecting the DNA polymerase that catalyzes the amplification reaction. Sample DNA dilution or purification may be used to minimize the effect of the inhibitors present, but these procedures can

reduce the sensitivity of detection. Regardless of the purification methods used an internal positive control should be incorporated into the PCR assay to avoid false negatives and determine whether the samples contain PCR inhibitors. In the present study 32 of 48 fungal DNA samples assayed contained PCR inhibitors, including all *A. flavus* isolates tested.

The real-time PCR primer and probe set developed in the current study for the rapid detection and enumeration of *A. flavus* in pure culture was validated thoroughly for cross-reactivity with DNA from target and nontarget organisms. QPCR analysis with TaqMan® fast chemistry technology can provide accurate quantitation of target nucleic acid sequences present in a sample with decreased analysis time, allowing for completion of PCR amplification in 35 min. The information obtained with this assay regarding absence (or presence) and concentration of *A. flavus* is expected to have use in indoor air quality monitoring and quality control of agricultural commodities and feed and can assist in the decision to analyze samples further for aflatoxins. The results of this research demonstrate the capabilities of QPCR for the enhanced detection and enumeration of fungi of significance to human health. Additional studies designed for the detection of *A. flavus* in food and environmental samples and the removal of PCR inhibitors would establish the field applicability of this assay. In the case of field samples, environmental sampling and processing protocols must consider methods that yield a liquid sample (e.g. homogenized food/feed material, swab, sponge) that is processed and analyzed by PCR without the need for cultivation. In summary the use of QPCR for the detection and enumeration of *A. flavus* provides enhanced detection capabilities that can be used to determine the level of contamination, or potential human exposure or to correlate spore measurements with toxin concentrations.

ACKNOWLEDGMENTS

The authors thank Joanne L. Henry for her valuable technical assistance. The research described in this article was financed in part by an Applied Research Initiative Award, University of Nevada at Las Vegas.

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