

Real-time PCR for detection of the *Aspergillus* genus

Marian D. Goebes,^{*a} Lynn M. Hildemann,^a Elmira Kujundzic^b and Mark Hernandez^b

Received 3rd January 2007, Accepted 2nd April 2007

First published as an Advance Article on the web 25th April 2007

DOI: 10.1039/b618937g

Aspergillus is a genus of mold that has strong indoor sources, including several species capable of acting as opportunistic pathogens. Previous studies suggest that *Aspergillus* could serve as an indicator for abnormal mold growth or moisture, making it an important genus for environmental monitoring. Here, a quantitative polymerase chain reaction (qPCR, or real-time PCR) assay is presented for *Aspergillus*. The assay shows good specificity for the genus, detecting all *Aspergillus* species tested, although a few non-*Aspergillus* species are also amplified. Sensitivity testing demonstrates that DNA representing one conidium can be detected. A validation study compared qPCR results against direct microscopy counts using *A. fumigatus* conidia aerosolized into a laboratory chamber. The assay was then used to quantify *Aspergillus* in indoor air samples, demonstrating its utility for environmental monitoring. Analysis of a small number of clinical sputum samples showed complete agreement with culturing results.

Introduction

Exposure to mold can elicit allergic reactions in fungal-sensitive individuals, who comprise roughly 10% of the total population and 40% of asthmatics.¹ *Aspergillus* is a particularly harmful genus of mold, including several species that are opportunistic pathogens capable of infecting immunocompromised individuals.² As judged by conventional enrichment surveys, the *Aspergillus* genus is also one of the most common genera of mold found in the indoor environment.^{3,4} This is of concern, because in the U.S., the average person spends 92% of their time indoors.⁵

Detection of *Aspergillus* in environmental and clinical samples is important, for monitoring indoor air quality and diagnosing infection, respectively. However, quantitative detection of *Aspergillus* remains challenging. Culture detection is time and labor intensive, and the selection of media leads to a bias in terms of which species may grow. Quantitative polymerase chain reaction (qPCR), or real-time PCR, is proving to be a promising tool; a well-designed assay can have high sensitivity, and the choice of primer and probe sequences allows for a range of specificity. Furthermore, the entire method of DNA extraction, clean-up and quantification by qPCR can be completed in less than a day.

Many mold conidia commonly detected indoors are from genera that primarily grow outdoors—the conidia then enter indoors *via* ventilation. Consequently, the indoor concentrations of conidia from outdoor molds could respond differently to environmental conditions or events than concentrations from mold genera that can readily grow indoors. This is supported by results from a culture-based study measuring

conidia counts in house dust, which found that *Cladosporium* levels were not or were only weakly correlated with levels of *Aspergillus* and *Penicillium*.⁶ Genus-level detection is advantageous for environmental monitoring, because it focuses on a group of similar species, but is more comprehensive than measuring a single organism.

Existing qPCR assays are panfungal,⁷ or detect one species of *Aspergillus*.^{8–11} A qPCR assay exists for the detection of the *Penicillium* and *Aspergillus* genera in environmental samples.¹² Yet although *Penicillium* has indoor sources, it appears to have stronger outdoor sources. An extensive study of airborne fungi in the U.S. found that, when detected in samples, *Penicillium* had a median concentration of 30 colony forming units (CFU) m⁻³ in indoor air samples, *versus* 50 CFU m⁻³ in outdoor air samples.¹³ In contrast, *Aspergillus* was found to have a median concentration of 20 CFU m⁻³ in both indoor and outdoor samples.¹³ While these concentration differences are based on recovery by culturing, total counts may follow a similar trend.

Results from previous studies suggest that relative concentration differences of *Aspergillus* could serve to indicate abnormal mold growth or moisture. The prevalence of *Aspergillus* conidia has been found to be higher in damp residences than in comparable dry settings.¹⁴ Burge *et al.*⁴ views relatively high, yet episodic, culture-based recovery of *Aspergillus* from office building air as an indication of a local indoor source of mold. A study seeking to identify moisture indicator fungi (MIF) included ten species of *Aspergillus*.¹⁵ Thus, genus-level specificity for *Aspergillus* is desirable for indoor environmental sampling, particularly if the goal is to focus on indoor sources of molds, or to assess if there is abnormal mold growth. Yet the close genetic similarity of *Aspergillus* with the *Penicillium* genus makes the development of an assay specific to *Aspergillus* difficult.¹⁶

Although previous qPCR assays have claimed *Aspergillus* specificity, these were designed expressly for clinical use. For example, the primers and probe in the real-time PCR assay presented by Kami *et al.*¹⁷ match sequences in the *Penicillium*

^aStanford University, Civil and Environmental Engineering Dept, Terman Engineering Center B13, Stanford, CA 94305, USA. E-mail: mdgoebes@stanfordalumni.org; Fax: +1 650 725 3162; Tel: +1 650 723 0315

^bUniversity of Colorado at Boulder, Dept of Civil, Environmental and Architectural Engineering, Boulder, CO 80309, USA

chrysogenum and *Penicillium brevicompactum* species exactly. Similarly, the probe specific for *Aspergillus* spp. in the PCR assay presented by Einsele *et al.*¹⁸ exactly matches the sequences in these two species. These *Penicillium* species are commonly found in indoor environments, making such assays unable to give *Aspergillus* genus-specific results in typical indoor settings.

For diagnosis of infection in clinical samples, sensitive and reliable detection is critical, because early initiation of anti-fungal treatment reduces the high mortality rate in infected patients.¹⁸ Several qPCR assays have been developed to detect *A. fumigatus*,^{8–10} the species responsible for 80–90% of all cases of invasive aspergillosis.¹⁹ However, other species of *Aspergillus* are also capable of causing invasive aspergillosis (e.g., *A. flavus*, *A. terreus*, *A. niger*, *A. nidulans*).¹⁹ Alternatively, panfungal assays do not discriminate among genera such as *Candida* and *Aspergillus*, which indicate the onset of markedly different diseases.

Like the assays presented by Kami *et al.*¹⁷ and Einsele *et al.*,¹⁸ this assay would detect all medically important *Aspergillus* species without detection of *Candida* species. This assay has the additional advantage of not detecting *Penicillium* species as strongly, thus making contamination during sample processing less likely. Loeffler *et al.*²⁰ indicate that clinical samples are occasionally contaminated by airborne fungal conidia during processing, leading to false positive results.

We report here the development and optimization of a rapid, reliable, and sensitive method for quantifying *Aspergillus* in environmental and clinical samples. Validation includes comparison with direct microscopy using impinger collected samples of laboratory chamber air, and with traditional testing methods using clinical sputum samples. *Aspergillus* was also quantified in indoor air samples.

Based on a literature review, this appears to be the first published qPCR assay that focuses its detection on the *Aspergillus* genus. Despite the detection of a few non-*Aspergillus* species, the ability of the assay to quantify a group of related species that can cause serious health problems and that may indicate abnormal indoor mold growth makes it a useful tool for environmental monitoring.

Materials and methods

Strains used

A list of strains used for sequence alignment is presented in Table 1a. The *Aspergillus* and non-*Aspergillus* strains used for experimental testing are presented in Table 1b and 1c, respectively—strains are listed in order of decreasing homology with

Table 1 (a) Strains used for sequence alignment, (b) strains used for experimental testing of target species and (c) strains used for experimental testing of potentially interfering species

Species	Strain ^a	Accession no. ^b
(a) Strains used for sequence alignment		
<i>Aspergillus fumigatus</i>	ALI 57	AF548063
<i>Aspergillus fumigatus</i>	UPSC 1771	AF548061
<i>Aspergillus fumigatus</i>	UPSC 2006	AF548062
<i>Aspergillus niger</i>	UPSC 1769	AF548064
<i>Aspergillus ochraceus</i>	UPSC 1983	AF548065

Table 1 (continued)

Species	Strain ^a	Accession no. ^b
<i>Aspergillus nidulans</i>	ATCC 10074	AB008403
<i>Penicillium marneffei</i>	AZN 747	N/A
<i>Aspergillus flavus</i>	UPSC 1768	AF548060
<i>Aspergillus terreus</i>	CBS 106.25	X78540
<i>Aspergillus penicilloides</i>	ALI 231	AF548066
<i>Aspergillus versicolor</i>	UPSC 1532	AF548069
<i>Aspergillus versicolor</i>	UPSC 2027	AF548068
<i>Aspergillus silvaticus</i>	ALI 234	AF548067
<i>Eurotium herbariorum</i>	ALI 216	AF548072
<i>Paecilomyces variotii</i>	UPSC 1651	AF548080
<i>Paecilomyces variotii</i>	UPSC 1766	AF548081
<i>Penicillium glabrum</i>	ALI 218	AF548090
<i>Penicillium brevicompactum</i>	ALI 321	AF548085
<i>Cladosporium cladosporioides</i>	UPSC 1657	AF548071
<i>Cladosporium cladosporioides</i>	ALI 50	AF548070
<i>Trichoderma viride</i>	ALI 210	AF548104
<i>Trichoderma harzianum</i>	ALI 232	AF548100
<i>Penicillium chrysogenum</i>	ALI 229	AF548086
<i>Penicillium chrysogenum</i>	UPSC 2020	AF548087
<i>Penicillium commune</i>	IBT 15141	AF548089
<i>Penicillium commune</i>	CBS 343.51	AF548088
<i>Penicillium italicum</i>	UPSC 1577	AF548091
<i>Paecilomyces lilacinus</i>	UPSC 1722	AF548079
<i>Ulocladium botrytis</i>	UPSC 3539	AF548106
<i>Ulocladium botrytis</i>	CBS 173.82	AF548105
<i>Stachybotrys chartarum</i>	ATCC 9182	AY489682
<i>Fusarium culmorum</i>	UPSC 1981	AF548073
<i>Microdochium nivale</i>	UPSC 3273	AF548077
<i>Candida albicans</i>	CBS 562	AF114470
<i>Saccharomyces cerevisiae</i>	ALI 308	AF548094
<i>Rhizopus microsporus</i>	UPSC 1758	AF548092
<i>Rhizopus rhizopodiformis</i>	NRRL 28630	AF113439
<i>Geotrichum candidum</i>	IFO 4599	AB000652
<i>Mucor plumbeus</i>	UPSC 1492	AF548078
<i>Wallemia sebi</i>	UPSC 2502	AF548108
<i>Wallemia sebi</i>	ALI 158	AF548107

(b) Strains used for experimental testing of target species

<i>Aspergillus fumigatus</i>	NRRL 163
<i>Aspergillus niger</i>	NRRL 326
<i>Aspergillus nidulans</i>	NRRL 187
<i>Aspergillus flavus</i>	NRRL 1957
<i>Aspergillus terreus</i>	NRRL 260
<i>Aspergillus versicolor</i>	NRRL 227

(c) Strains used for experimental testing of potentially interfering species

<i>Paecilomyces variotii</i>	NRRL 1115
<i>Penicillium glabrum</i>	NRRL 766
<i>Penicillium brevicompactum</i>	NRRL 859
<i>Cladosporium cladosporioides</i>	ATCC 6721
<i>Penicillium chrysogenum</i>	NRRL 807
<i>Stachybotrys chartarum</i>	ATCC 9182
<i>Candida albicans</i>	NRRL Y-12983
<i>Geotrichum candidum</i>	NRRL Y-552
<i>Escherichia coli</i>	ATCC 11303 ^c
<i>Escherichia coli</i>	ATCC 33694 ^d
<i>Enterobacter cloacae</i>	ATCC 13047

^a ALI = Arbetslivsinstitutet, Umeå, Sweden; UPSC = Uppsala University Culture Collection of Fungi, Uppsala, Sweden; ATCC = American Type Culture Collection, Manassas, VA, USA; AZN = Collection of the Department of Medical Microbiology, Radboud University, Nijmegen, The Netherlands; CBS = Central bureau voor Schimmelcultures, Baarn, The Netherlands; IBT = Culture Collection of Fungi, Technical University of Denmark, Lyngby, Denmark; NRRL = Agricultural Research Service Culture Collection, Peoria, IL, USA; IFO = Institute for Fermentation, Osaka, Japan. ^b N/A: not available in NCBI database. ^c Strain used in experimental specificity testing. ^d Strain used in validation study.

the assay, based on their genetic sequence. All work involving *A. fumigatus* and clinical specimens was performed in biosafety level-2 laminar flow biosafety cabinets. The same strains of *A. niger*, *A. fumigatus* and *Geotrichum candidum* used for experimental specificity testing were used for the validation study, and as standards for the air samples and clinical samples. Two different strains of *Escherichia coli* were used for the experimental specificity testing and validation study. *Aspergillus*, *Penicillium*, and *Paecilomyces* spp. were grown on malt extract agar and Sabouraud agar. *Cladosporium cladosporioides* and *Saccharomyces candidum* were grown on potato dextrose agar and cornmeal agar, respectively. Yeasts (*G. candidum* and *Candida albicans*) were grown on Sabouraud agar and yeast malt agar. All fungal species were grown at room temperature (approximately 25 °C) for 7–14 days. For the specificity tests, *Escherichia coli* and *Enterobacter cloacae* were grown in Luria–Bertani (LB) and nutrient media, while the *E. coli* for the validation study was grown in tryptic soy broth. All bacteria were grown at 37 °C for 1 day.

Conidia collection

Conidia were collected by rolling a cotton swab moistened with sterilized, deionized water across a sporulating culture, and dipping the swab into a 1% Triton solution. After homogenization on a shaker, the cell density was determined using a Bright-Line hemacytometer cell chamber (Hausser Scientific, Horsham, PA, USA).

DNA extraction and purification

The procedure used to pellet the conidia varied according to the type of sample (*i.e.*, impinger sample for the validation study, laboratory culture for specificity and sensitivity testing, air sample, or clinical specimen). The pellet was resuspended in 0.5 ml extraction buffer (50 mM Tris-HCl, pH 7.5; 50 mM EDTA; 2% SDS; 1% Triton-100), and transferred to a bead-beating tube (Sarstedt Inc., Newton, NC, USA), to which 0.3 g of sterilized zirconia/silica beads (BioSpec Products, Inc., Bartlesville, OK, USA) were added. Adding carrier cells before DNA extraction greatly increased DNA yield during extraction and purification when a small number of conidia (*i.e.*, numbers typical for environmental samples) were present, as suggested by R. Haugland (personal communication). Thus, *G. candidum* were added to samples in the validation study, and when processing air and clinical samples. Carrier cells were not used in sensitivity or specificity testing, because the large numbers of *Aspergillus* conidia processed in these tests made carrier cells unnecessary, and because experimental quantification using DNA concentrations required the use of pure cultures. Carrier cells were collected according to the method described for conidia collection. Solutions containing 2×10^6 *G. candidum* cells were pipetted into centrifuge tubes and centrifuged at $16000 \times g$ for 10 minutes. The resulting supernatant was discarded, and the pellet was added to the specimen pellet. To ensure that the carrier cells did not yield a false-positive result, qPCR was performed with the DNA resulting from the lysis and purification of 2×10^6 *G. candidum* cells.

Bead-beating was performed with a Mini-Beadbeater (BioSpec Products, Inc., Bartlesville, OK, USA) for 2 minutes at maximum speed. Samples were immediately transferred to a 65 °C water bath for 10 minutes. 160 µl of buffer AP2 from the Qiagen Plant Kit (Qiagen, Inc., Valencia, CA, USA) was added to the sample, which was then vortexed and incubated on ice for 5 minutes. The solution was centrifuged for 5 minutes at $16000 \times g$, and the supernatant discarded. The remainder of the procedure followed the Qiagen Plant Kit protocol, with two 50 µl volumes of buffer AE used to elute DNA in the final step. This extraction–purification protocol, adapted from Wu *et al.*,²¹ was found to give a consistent and high yield of 0.5 µg DNA from 10^7 *A. flavus* conidia.

Primer/probe design

A conventional PCR assay published by Melchers *et al.*²² for the detection of *Aspergillus* utilizing the 18S ribosomal RNA gene was first tested using SYBR Green qPCR and the authors' cycling parameters²² under a range of primer and MgCl₂ concentrations on an Applied Biosystems, Inc. (Foster City, CA, USA) 7000 Sequence Detection System (“ABI machine”). However, the assay was found to be too insensitive for processing environmental samples. Based on sequence alignment of these primers with several *Aspergillus* and *Penicillium* species, the specificity of the assay came entirely from the reverse primer, “Asp2” (5'-ACCCCCCTGAGC-CAGTCCG-3'). Consequently, a new assay was designed with a new forward primer and TaqMan probe, but retaining Asp2 as the reverse primer.

New forward primer candidates were designed using Net-Primer software from PREMIER Biosoft International (Palo Alto, CA, USA) to check for the likelihood of formation of dimers, and tested using SYBR Green qPCR on the ABI machine. The new forward primer selected, named “AspG” (5'-GCCAGCGAGTACATCACCTTGG-3'), showed particularly high sensitivity and included a basepair mismatch with most *Penicillium* species at its 3' end.

Primer Express (ABI) was used to identify potential probe sequences with a melting temperature (T_m) desirable for TaqMan qPCR. Candidate sequences were tested for dimer formation using NetPrimer and laboratory testing, and “Prb137” (6-FAM-5'-ACGTCCCTGCCCTTGTACACACCG-3'-TAMRA) was chosen as the TaqMan probe.

qPCR reaction

TaqMan qPCR analysis was performed on two machines: the ABI machine, and an MJ Research DNA Engine Opticon Continuous Fluorescence Detector (the “MJ Research machine,” Bio-Rad Laboratories, Inc., South San Francisco, CA, USA). Primers were synthesized by Operon Biotechnologies, Inc. (Huntsville, AL, USA) and purified *via* high performance liquid chromatography (HPLC). The TaqMan probe was synthesized by ABI. Reaction chemistry and cycling conditions varied for the different machines, based on the manufacturers' recommendations. For the MJ Research machine, each reaction contained 25 µl 2x iTaq Supermix, 500 nM of each primer, and 200 nM of probe. For the ABI machine, each reaction contained 25 µl 2x Universal Master Mix, 900 nM of

each primer, and 250 nM of probe. For both machines, sterilized, deionized water was added to give a total reaction volume of 50 μl . For specificity and sensitivity testing, 2 μl of DNA template was added; for the validation tests, air samples, and clinical samples, 5 μl of DNA template was added to each well. All qPCR runs included a no template control (NTC) reaction, in which a volume of sterile water equivalent to the template used for that run was added to a well; data from runs in which the NTC amplified were discarded. Cycling conditions for the MJ Research machine were: 3 minutes at 95 $^{\circ}\text{C}$; and 40 cycles of 15 seconds at 95 $^{\circ}\text{C}$ followed by 45 seconds at 56 $^{\circ}\text{C}$. For the ABI machine, cycling conditions were 2 minutes at 50 $^{\circ}\text{C}$; 10 minutes at 95 $^{\circ}\text{C}$; and 40 cycles of 15 seconds at 95 $^{\circ}\text{C}$, followed by 45 seconds at 56 $^{\circ}\text{C}$. The default threshold fluorescence value was chosen for the ABI machine. Because the MJ Research platform does not have default threshold settings, an operating fluorescence threshold was chosen as that level corresponding to the beginning of the log-linear response for all amplifying samples in the experimental set, and above the highest level of fluorescence attained by the blank.

Validation experiments

Validation experiments were conducted using a small pilot-scale chamber, as described by Peccia *et al.*²³ After purging the chamber with purified air for at least 12 hours, airborne fungal conidia were continuously generated for 20 minutes. No ventilation was provided during the aerosolization and sampling period, but a 42 W fan (Caframo Ltd, Wiaraton, Canada) operating in the chamber ensured mixing. After aerosolization was stopped, airborne fungal conidia were sampled for 10 minutes with 3 swirling liquid impingers (BioSampler, SKC Inc., Eighty-Four, PA, USA). Between experiments, the chamber was cleaned with 10% bleach and purged with clean air overnight.

Pure cultures of *A. fumigatus* and *A. niger* were used for the validation study. Just before aerosolization, fungal conidia were removed from plate surfaces by aseptic shaking with 3 mm glass beads (Fisher Scientific, Pittsburgh, PA, USA) and were suspended in phosphate buffered saline (PBS: 11.9 mM phosphate, 137 mM NaCl, 2.7 mM KCl; pH = 7.4) with 0.1% Tween 80 (Sigma, St. Louis, MO, USA) for a total volume of 50 ml. Conidia stocks were homogenized by sonication in ice water and transferred to a six-jet collision nebulizer (CN 25, BGI Inc., Waltham, MA, USA), which was operated at 138 kPa (20 psi). Air supplying the nebulizer was purified by carbon filtration, desiccated by filter apparatus (Model 3074, TSI Inc., St. Paul, MN, USA) and particle filtered by a 0.2 μm filter (Gelman Bacterial Air Vents, Pall Corporation, East Hills, NY, USA).

Before each experiment, the impingers were washed with ethanol and deionized water, and autoclaved. The impingers were filled with 20 ml of sterile PBS for bioaerosol collection and collected bioaerosols simultaneously for 10 minutes at a flow rate of 12 liters per minute (LPM) using a high-flow sampling pump (Gas Manufacturing Inc., Benton Harbor, MI, USA) regulated with rotameters (Cole Parmer Instrument Company, Vernon Hills, IL, USA). A fourth impinger, which

served as a blank, was present inside the chamber during the time of sampling, but was not connected to the pump. After each sampling collection period, the chamber was opened, impingers removed, and their reservoir contents split for analysis with direct microscopy and qPCR.

For qPCR analysis, impinger solutions were centrifuged at $3200 \times g$ for 30 minutes, and the supernatant discarded. After DNA extraction and purification of the pellet, qPCR was performed on the MJ Research machine.

Number concentrations of total fungal conidia were determined using direct microscopy. The impinger solution was stained with conventional DNA intercalating agent, SYTO 9 (5 μM , Molecular Probes, Eugene, OR, USA) for two hours, and then passed through 25 mm, 1.0 μm (pore diameter) polycarbonate membrane filters (Poretics Inc., Livermore, CA, USA). Fungal conidia retained on the filter surface were mounted using low fluorescence immersion oil containing an antifadent (CitiFluor Ltd, Leicester, UK), and examined under $1100\times$ magnification using a Nikon Eclipse E400 epifluorescent microscope fitted with a mercury lamp and polarizing filters. Ten random fields were counted per slide. All counts were reported as the average of all microscopic fields counted.

Two sets of duplicate experiments performed in the chamber were analyzed using both methods. The first set used a nebulizer solution of 1.6×10^7 *A. fumigatus* conidia ml^{-1} , as calculated by direct microscopy, and the second used 3.3×10^7 *A. niger* conidia ml^{-1} . After the first experiment of each set, the nebulizer solution was returned to a sterile bottle, and a small amount of PBS (about 5 ml, representing 10% of the total volume) was added to return the volume to 50 ml for the second duplicate experiment. In general, impinger solutions were evenly split for direct microscopy and qPCR analysis; however, in one case, a greater volume was used for direct microscopy to remain above the detection limit of this method.

For one *A. fumigatus* experiment, a portion of each of the three impinger solutions was extracted prior to splitting the samples for the two analytical methods. These portions were combined and used to create a serial dilution from 10^{-1} to 10^{-5} . Because of direct microscopy detection limits, only the numbers of conidia in the 10^{-1} and 10^{-2} solutions could be visually confirmed; all other concentrations were calculated using the 10^{-2} solution, assuming perfect dilutions.

For one *A. niger* experiment, the solution for qPCR analysis was split into three equal aliquots to evaluate the standard deviation of the extraction–purification process. Triplicate qPCR reactions were then performed for each of the three samples to evaluate the standard deviation of the qPCR.

A final set of experiments used concentrations of *A. fumigatus* representing more typical indoor concentrations, both to confirm the assay's ability to detect ambient levels, and to evaluate the effect of a non-*Aspergillus* species. One chamber experiment used a diluted portion of the *A. fumigatus* nebulizer solution (approximately 160 conidia ml^{-1}). A second chamber experiment used this solution, but with the addition of 5 ml of 10^4 *E. coli* cells ml^{-1} . Samples were analyzed by qPCR, but direct microscopy was not performed because the solution was below the detection range of this method.

Specificity testing

Thousands of fungal species exist, and our approach to specificity testing was to focus on species that are commonly detected in indoor air samples, and closely analogous to the assay. A literature review of research discussing common airborne fungi^{13,16,22} generated a list of fungi commonly detected in air, and a few common bacteria.

To select which of these species to test, sequence alignment was performed comparing each species in the list with the assay, using the MegAlign 5.03 software program (DNA-STAR, Inc., Madison, WI, USA) and National Center for Biotechnology Information (GenBank) in July 2005. The 18S ribosomal RNA genes from 41 strains of 32 species, including 13 strains of 10 *Aspergillus* species and 28 strains of 22 non-*Aspergillus* species, were aligned with the primer/probe set. The 18S ribosomal RNA gene of *A. fumigatus* was used for the primer/probe sequence.

Using the MegAlign results, species were listed in order of decreasing homology with the primer/probe set. Species were tested experimentally starting at the top of the list (*i.e.*, most homologous with the assay), using 1 ng DNA from the tested organism, extracted and purified from conidia as described above. The DNA concentration of the resulting eluate was measured using a DynaQuant 200 fluorometer (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA). A portion of this eluate was then diluted with sterile, deionized water so that 2 μ l of the solution contained 1 ng DNA.

The DNA solutions were then analyzed with the assay by qPCR on both the MJ Research and the ABI machines. Organisms were tested in order of decreasing homology until consistent non-detect values were achieved; after this point, a few additional organisms were tested to verify their non-detect status. For species in Table 1a which were found to have identical sequences over the primer/probe regions (*e.g.*, *A. fumigatus* and *A. ochraceus*; *P. chrysogenum*, *P. commune* and *P. italicum*), only one species was tested. A total of 6 *Aspergillus* species and 10 non-*Aspergillus* species were tested. Specificity testing was performed on both machines, because different DNA polymerases and methods for calculating the threshold fluorescence values were used, and because the machines differ in temperature ramping rates.

Sensitivity testing

To assess the detection limit of the assay, a dilution series was made with DNA extracted from *A. flavus* conidia. Conidia collection, and DNA extraction, purification, and quantification was performed as described for "Specificity testing". QPCR was performed on the series using the ABI machine. The following equation, recommended by ABI, was used to calculate amplification efficiency, *eff.* (perfect efficiency = 1.0):

$$\text{eff.} = [10^{(-1/\text{slope})} - 1]$$

where slope = slope of the standard curve (cycle threshold [C_T] vs. number of conidia) in log-linear form.

Air samples

Air samples were collected in indoor environments in the Palo Alto, CA, USA area in 2005 and 2006. The majority of

sampling sites were offices, but a university classroom, a residential bathroom, and a hospital were also sampled. Air samples were taken for approximately 4 hours at a height of 1 meter, using 47 mm diameter, 0.45 μ m (pore diameter) Durapore membrane filters (Millipore Corp., Billerica, MA, USA) with Gelman filter holders (Pall Corporation), and a 115 V pump (Medo USA, Inc., Hanover Park, IL, USA). Flow rates were measured using a Bubble Generator (Gilian Instrument Corp., West Caldwell, NJ, USA), and averaged 10 LPM. A custom-built cyclone, designed based on specifications from John *et al.*,²⁴ was used upstream of the filters to select for particulate matter $\leq 5 \mu$ m in diameter ($PM_{2.5}$).

Filters were transferred to 15 ml Falcon tubes (BD Biosciences, San Jose, CA, USA) and stored at 4 °C until ready for processing. For extraction, 2.5 ml of the buffer used in DNA extraction was added to the tube. The tube was vortexed for 5 minutes, the filter removed, and the tube centrifuged at 2850 $\times g$ for 30 minutes. Supernatant was removed so that 0.5 ml of solution remained. The pellet was resuspended in this solution and transferred to a bead-beating tube. DNA extraction and purification were performed, and the ABI machine was used for qPCR. Air samples were processed in a batch that included a blank filter to check for contamination during DNA extraction and purification. If the blank amplified in qPCR, the air samples processed in this batch were discarded.

The following equation was used for quantification of qPCR results:

$$\text{Unknown value} = \text{Std value} \times [(1 + \text{eff})^{\text{Std } C_T} / (1 + \text{eff})^{\text{Unknown } C_T}]$$

where Unknown value = initial number of *A. fumigatus* conidia equivalents in the air sample, Std value = initial number of *A. fumigatus* conidia in the standard (as determined by the hemacytometer), Std C_T = cycle threshold achieved by the standard for that qPCR run, and Unknown C_T = cycle threshold achieved by the air sample. Amplification efficiency was calculated using a standard curve generated with a dilution series of *A. fumigatus* conidia, and the same equation for efficiency was used for sensitivity testing. One standard with a value of 100 *A. fumigatus* conidia (a typical value for an air sample) was included in every set of samples analyzed by qPCR to ensure that the qPCR run behaved as predicted by the standard curve, and to account for machine differences among runs. Inspection of the C_T values for different 100 *A. fumigatus* conidia standards indicated little variation among qPCR runs (standard deviation of 0.40 cycles). *A. fumigatus* was used for the standards, because it is commonly found in indoor air.¹³

Clinical specimens

A small number of sputum specimens were also tested to explore the assay's potential for clinical work. Sputum specimens were obtained from patients at risk for invasive aspergillosis, including cystic fibrosis and bone marrow transplant patients, as required by their physicians. Appropriate institutional approvals were obtained.

In a clinical laboratory at Stanford Hospital that conducted the culturing, purulent sections of expectorated sputum or

aspirates were chosen with wooden sticks and inoculated to Sabouraud's Dextrose, Mycobiotic, CHROMagar Candida (all from BD Diagnostics Products, Sparks, MD, USA) and Potato Flake agar (prepared in-house). Plates were streaked for isolation and incubated at 30 °C for 3 weeks. Plates were examined daily and mold colonies were presumptively identified by morphology as visualized in a stereomicroscope, and microscopically by examination of lacto-phenol cotton blue adhesive tape preparations. Slide cultures were prepared for molds that could not be identified by initial morphological observations. For specimens that tested positive *via* culturing, the remainders of the specimens were stored at 4 °C, along with a random sampling of specimens that tested negative, to create a blinded batch for qPCR analysis by a separate research laboratory.

For qPCR analysis, DNA purification from sputum specimens began with the approximation of sputum volume, and the addition of an equal volume of Sputolysin reagent (Calbiochem, San Diego, CA, USA). Samples were vortexed for 30 seconds, allowed to stand at room temperature for 15 minutes, and vortexed again for 30 seconds. Solutions were centrifuged at 16 000 × *g* for 30 minutes, the supernatant was discarded, and DNA extraction and purification was done on the pellet. QPCR was performed on the ABI machine. As with the air samples, a blank was processed with each batch of sputum samples to check for contamination during extraction and purification, and the blanks did not amplify during qPCR.

Once extracted, sputum samples were quantified using the same analytical paradigm as the air samples. However, the sputum matrix is particularly purulent, and experiments conducted by spiking known numbers of *A. fumigatus* conidia into uninfected sputum indicated that the high concentration of background human DNA inhibited the qPCR reaction. Consequently, inhibition was taken into account in the analysis of the sputum samples. *A. fumigatus* was used for calibrating clinical specimens, because this species is found in the majority of infected patients.²⁵ The standard used in each run, and the standards used in the standard curve for determination of amplification efficiency, were made by injecting a known number of *A. fumigatus* conidia into sputum that had previously been found to test negative for *Aspergillus* both by culturing and qPCR. Negative samples from nine patients were pooled to account for differences among individuals' specimens.

Results

Validation

Results of the chamber experiments calibrating qPCR C_T values to direct microscopy results are presented in Fig. 1. This validation curve has an R^2 value of 0.97, indicating the assay can accurately quantify the number of conidia in a sample over a 5 log concentration range. Residuals were found to be normally distributed, indicating that the remaining variation was random. By splitting impinger contents into three aliquots, the variance of the extraction–purification process was found to be 0.5 qPCR cycles, and 0.2 cycles for the qPCR process. For the two experiments investigating the

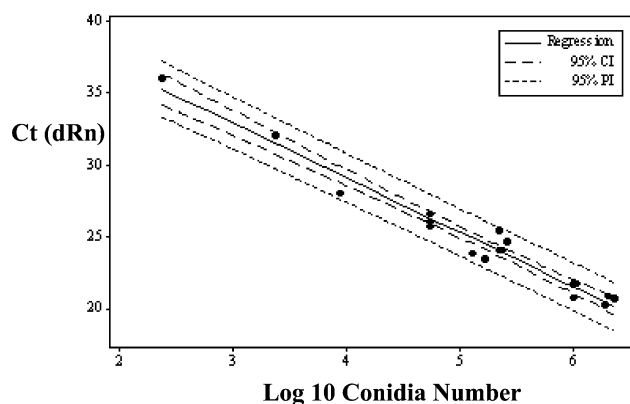


Fig. 1 Validation study results. Threshold cycle (C_T) determined by qPCR and conidia number determined by direct microscopy. $R^2 = 0.97$, $C_T = 44.34 - 3.81 \log_{10}$ conidia. The standard deviation of the direct microscopy counts was used to construct error bars. For the impinger dilution series, the standard deviation for the 10^{-3} and 10^{-4} dilution points was found by scaling down the standard deviation for the 10^{-2} dilution point (the last solution counted). Standard deviation due to pipetting was neglected, because a pipetting calibration experiment indicated that the standard deviation for the 10-fold dilution is 0.07%, compared with the 19% standard deviation for the cell count. Consequently, it is unlikely that the error bars would be significantly affected by pipetting error.

effect of another organism's presence, the average C_T values among triplicates were 34.9 and 35.6 when *E. coli* were absent and present, respectively. The 0.7 C_T difference between these means is less than the 0.8 C_T difference between the means of the qPCR results from the first set of duplicate chamber experiments (where using a pure solution of 1.6×10^7 *A. fumigatus* conidia ml^{-1} yielded means of 20.5 and 21.3); and less than the 1.1 C_T difference in means of the qPCR results from the second set of duplicate chamber experiments (where using a pure solution of 3.3×10^7 *A. niger* conidia ml^{-1} yielded means of 24.7 and 23.6†). Thus, the difference in means between the *A. fumigatus* experiments performed with *E. coli* present and absent is comparable in magnitude to the differences found for duplicate chamber experiments performed with pure cultures. The C_T values from the experiments conducted to investigate the effect of the presence of *E. coli* were also similar to the theoretical C_T predictions from the validation curve. Using direct microscopy of the original *A. fumigatus* nebulizer solution, and assuming perfect dilution, the samples contained approximately 85 conidia, corresponding to an expected C_T of 35.7 according to the log-linear calibration and associated extrapolation.

Specificity

Specificity results, presented in Table 2, indicate that sensitivity was similar, but not identical, among the *Aspergillus* species. Less interspecific variation was seen on the MJ Research machine than on the ABI machine. Table 2 shows that on both machines, the assay strongly amplifies *Paecilomyces variotii* and weakly amplifies *Penicillium glabrum*. The

† Results from the impinger sample that was split into three aliquots were not included in the calculation of this mean.

Table 2 Specificity results. Species listed in order of decreasing homology with assay

Species	No. of Bp mismatches (No. at 3' end)	Gap length	C _T for 1 ng DNA, ABI qPCR	C _T for 1 ng DNA, MJ research qPCR
<i>Aspergillus fumigatus</i>	1 (0)	0	20.9	20.8
<i>Aspergillus niger</i>	1 (0)	0	18.3	21.5
<i>Aspergillus nidulans</i>	1 (0)	0	19.4	21.3
<i>Aspergillus flavus</i>	2 (0)	0	20.4	21.3
<i>Aspergillus terreus</i>	2 (0)	0	18.2	21.6
<i>Aspergillus versicolor</i>	2 (0)	0	22.7	22.4
<i>Paecilomyces variotii</i>	1 (1)	0	22.3	20.6
<i>Penicillium glabrum</i> ^a	2 (1)	0	30.0	33.8
<i>Penicillium brevicompactum</i>	4 (2)	0	> 40	34.3
<i>Cladosporium cladosporioides</i>	7 (0)	0	> 40	> 40
<i>Penicillium chrysogenum</i>	6 (2)	0	> 40	> 40
<i>Stachybotrys chartarum</i>	5 (1)	2	> 40	> 40
<i>Candida albicans</i>	10 (0)	1	> 40	— ^b
<i>Geotrichum candidum</i>	14 (1)	2	> 40	> 40
<i>Escherichia coli</i>	NA ^c	NA ^c	> 40	> 40
<i>Enterobacter cloacae</i>	NA ^c	NA ^c	> 40	> 40

^a *Penicillium glabrum* is also known as *Penicillium frequentans*. ^b Specificity testing was not performed with this species on this machine. ^c NA = Not applicable. Bacteria do not have 18S ribosomal RNA genes, so sequence alignment was not performed.

MJ Research machine also weakly amplifies *Penicillium brevicompactum* while the ABI machine does not.

Sequence alignment indicates that the assay would strongly amplify *Penicillium marneffeii*; this species was not included in specificity testing, because, as explained in the Discussion, it has a very restricted habitat and its presence in an air sample is highly unlikely.

Anneal temperature experiments were performed on the ABI machine to find a temperature that would minimize amplification differences among *Aspergillus* species but still discourage amplification of other fungal genera. Although this assay amplified *Aspergillus* at annealing temperatures as high as 64 °C, this led to large sensitivity differences among the *Aspergillus* species, with *A. niger* having a C_T value > 5 cycles lower than *A. flavus*. At 64 °C, *P. variotii* continued to be amplified with an efficiency similar to *Aspergillus* species, and *P. glabrum* was also amplified, although with a reduced efficiency. Annealing temperatures below 56 °C created no greater uniformity in C_T responses from *Aspergillus* species; thus, 56 °C was chosen to strike a balance between hybridization stringency and the response of qPCR across the *Aspergillus* genus. This temperature reduced the C_T difference between *A. niger* and *A. flavus* to 2 cycles, and induced *P. glabrum* to amplify with a significantly reduced efficiency when compared with the median *Aspergillus* response. All TaqMan qPCR results presented here were obtained with an annealing temperature of 56 °C.

Specificity testing with *G. candidum*, the species used as carrier cells, was performed with 1 ng of DNA on both the ABI and MJ Research machines. Testing was also performed with DNA extracted from 2 × 10⁶ cells (the number of cells used when carrier cells were added) on the ABI machine. None of these amplified with the assay.

Sensitivity

Fig. 2a shows the amplification plot generated by a serial dilution series, and Fig. 2b shows the corresponding standard curve on the ABI machine. The assay reliably reported DNA

masses between 500 pg (the highest point in the dilution series) and 50 fg. Based on calibration using pure *A. flavus* conidia in a hemacytometer, and quantification of the extracted DNA for the highest point in the dilution series using a spectrophotometer (Model ND-1000, NanoDrop, Wilmington, DE,

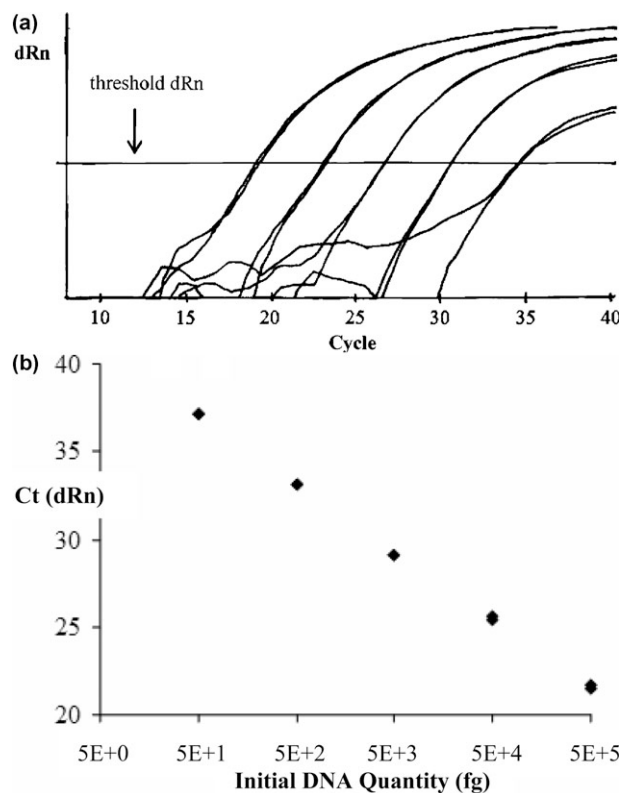


Fig. 2 (a) Detection of *A. flavus* using qPCR. Five-fold serial dilution of *A. flavus* DNA ranging from 500 pg to 50 fg and detection using the ABI machine. The horizontal line represents the threshold value for baseline corrected normalized fluorescence (dRn). (b) The corresponding standard curve. Each dilution point has two data points, although the values for these duplicates are so similar that they are indistinguishable on the plot.

USA), the DNA harvested ranged from 10^4 conidia (500 pg) to 1 conidium (50 fg). The purification method described here yielded approximately 100 μ l of DNA eluate; the qPCR reaction for air and clinical samples, and the validation study, used 5 μ l of this DNA solution (or 1/20 of the total) as a template. Thus, because the DNA mass of an individual conidium in the qPCR reaction effectively corresponds to 20 conidia in an optically calibrated sample (by dilution), the method should be capable of detecting 20 conidia in an uninhibited environmental sample.

The standard regression in Fig. 2b ($R^2 = 0.9995$) demonstrates a predictable log-linear response over a broad range of DNA concentrations. Using this standard curve, the amplification efficiency was found to be 82%. As judged by C_T values, *A. flavus* was chosen for the sensitivity test, because the assay reported a median response for this species relative to the other *Aspergillus* species tested here.

Air samples

Table 3 presents air sampling results. Samples were taken in duplicate, and each sample was run in duplicate qPCR reactions, yielding a total of four qPCR results for each measurement. For a measurement to be considered positive, all four reactions had to amplify. The standard deviation for each measurement, ranging between 0 to 35 *A. fumigatus* conidia equivalents m^{-3} , reflects the variation among the qPCR runs as well as sampling and extraction-purification variation between the air samples; variation from the sampling and extraction-purification was generally higher than qPCR variation from the same extract. Based on the standard regression of *A. fumigatus* conidia, the amplification efficiency was found to be 81% ($R^2 = 0.997$). Calibration was performed with pure laboratory cultures diluted in reagent grade solutions; consequently, potential PCR inhibition effects were not isolated from the quantification of conidia recovered in air samples.

Clinical specimens

Results from the sputum samples tested are presented in Table 4. As shown, a total of ten samples were analyzed. All samples

Table 3 Air sampling results

Location	<i>Aspergillus</i> conidia ^a m^{-3}	Std Dev. (conidia m^{-3})
Office 1	71	35
Office 2	3	1
Office 3	17	8
Office 4	44	3
Office 5	2	0
Office 6	7	6
Office 7	47	12
Office 8	4	1
Classroom	26	18
Academic Hallway	85	11
Residential Bathroom	261	31
Hosp. Wait. Room 1	9	6
Hosp. Wait. Room 2	50	31
Hospital Hallway 1	6	4
Hospital Hallway 2	18	4

^a Values are presented as equivalent numbers of *A. fumigatus* conidia.

Table 4 Sputum results: Comparison of qPCR with culturing

Sample ID	qPCR: Avg C_T	qPCR: C_T Std. Dev.	qPCR result: (<i>Aspergillus</i> conidia) ^a	Culturing result
1a	34.7	0.8	209	(+)
2a	>40	NA	ND	(-)
3a	>40	NA	ND	(-)
4a	>40	NA	ND	(-)
5a	>40	NA	ND	(-)
6a	>40	NA	ND	(-)
1b	>40	NA	ND	(-)
2b	>40	NA	ND	(-)
3b	36.6	0.7	82	(+)
4b	>40	NA	ND	(-)

^a Values are presented as equivalent numbers of *A. fumigatus* conidia. NA = Not applicable; ND = Not detected.

were run in duplicate qPCR reactions. Both methods detected samples 1a and 3b as positive, and the remaining samples negative. It was found that the best results were obtained when the full sputum sample available for qPCR analysis was processed. Using the standard curve generated by spiking *A. fumigatus* conidia into sputum, qPCR amplification efficiency was found to be 72%.

Discussion

The DNA sequence complementary to the TaqMan probe of this assay (Prb137) is present in many fungi. Thus, the probe does not provide additional specificity, but rather serves to confirm that the desired sequence has been amplified. The primers of this assay (AspG and Asp2) provide the specificity for the *Aspergillus* genus. Consequently, while the assay was designed for TaqMan qPCR use, the primers could be used alone in conventional PCR or SYBR Green qPCR, although another means should be used to confirm that the amplicon produced is the target (*Aspergillus*) sequence.

The sensitivity differences among *Aspergillus* could arise from two phenomena: (1) basepair mismatch location and annealing efficiency, and (2) intrinsic content of target DNA (copy number of target sequences/DNA template). None of the *Aspergillus* spp. evaluated here has a 3' basepair mismatch with the probe or either primer, but the various species have one or two basepair mismatches at different locations. For example, *A. niger* has 1 basepair mismatch with Asp2, while *A. flavus* has 2 mismatches with this primer. These differences may cause variations in annealing efficiency, leading to variations in amplification efficiency. The second possibility lies in the 18S gene copy numbers for each species. The rDNA copy number for *A. nidulans* was reported as 45 for a haploid cell²⁶ (i.e., 90 for diploid) but is unknown for other *Aspergillus* species.† It is possible that the various *Aspergillus* species have different rDNA copy numbers. (For example, Maleszka *et al.*²⁷ found a difference in rDNA copy numbers between two species of the same genus of yeast, *Kluyveromyces*.) At

† Although the genomes have been sequenced for *A. fumigatus* and *A. oryzae*, the number of 18S copies per genome is not readily available, as the ribosomal RNA array cluster is not part of the genome assembly for any of these species (A. Rokas, personal communication).

lower annealing temperatures, the differences in basepair mismatches may affect annealing efficiency only slightly, but varying copy numbers of 18S ribosomal RNA genes per genome (and therefore per conidia) would affect sensitivity across all annealing temperatures.

Sequence alignment and specificity experiments indicate that all *Aspergillus* analyzed were strongly detected, and that most non-*Aspergillus* species tested were not detected. The non-*Aspergillus* species that were amplified by the assay fell into two categories.

The first category includes species that were indicated by sequence alignment, or shown by specificity testing, to be strongly detected by the assay; that share the clinical significance of several *Aspergillus* species of being opportunistic pathogens; and that have been identified by other researchers as having genetic similarities with *Aspergillus*. *P. marneffeii*, the first of the two species in this category, is an emerging opportunistic pathogen endemic in Southeast Asia.²⁸ Approximately 8% of AIDS patients in Hong Kong are infected with *P. marneffeii*.²⁹ The species has also been shown to have a close genetic relationship with *Aspergillus*.²⁹ Sequence alignment performed here indicated that this species would amplify with sensitivity similar to *Aspergillus*. *P. marneffeii* has an extremely narrow range of habitat, and has thus far only been found as a pathogen infecting immunocompromised people and the bamboo rat of Southeast Asia,³⁰ and in soil samples from bamboo rat burrows.³¹ No reports have described the existence of a free-living stage of *P. marneffeii*, and it has never been reported in air sampling.²⁹ A study presenting a DNA probe for an enzyme immunoassay (PCR-EIA) designed to detect medically important *Aspergillus* species also detects *P. marneffeii*, and the authors state that the taxonomies of many molds are in a state of flux after the introduction of molecular taxonomic methods.³² *P. variotii* is the second species in this category, and it was strongly amplified by the specificity testing performed here. Besides acting as an opportunistic pathogen,³³ *P. variotii* has been listed as a species commonly found in indoor environments,³⁴ and in moisture-damaged schools.³⁵ A phylogenetic tree of the 18S gene indicates the species is very genetically similar to *Aspergillus*.¹⁶ Thus, the detection of *P. marneffeii* and *P. variotii* by the assay designed for *Aspergillus* detection is not surprising, and is beneficial for its potential uses in detecting opportunistic pathogens, and in monitoring molds that can grow well indoors.

The second category of non-*Aspergillus* species detected by the assay include species that were weakly amplified by the assay. Specificity testing with both the ABI and MJ Research machines showed weak amplification of *Penicillium glabrum*, and the MJ Research machine weakly amplified *Penicillium brevicompactum*. However, the sensitivity of the assay to these species (*P. glabrum* on both machines and *P. brevicompactum* on the MJ Research machine) is almost one-thousand-fold lower than what was observed for *Aspergillus*. Consequently, even a large number of conidia from these species in a sample would translate into a very small signal by the assay. From the perspective of applied microbiology for use in environmental or clinical settings, the assay is still useful despite the slight detection of the species in this second category. Another study presenting a variety of qPCR assays includes several assays

which detect non-target species, but at reduced sensitivities, so that they are detectable only when present in approximately 1000-fold or greater quantities than the target organisms.¹²

Possible reasons for the specificity discrepancy between machines for *P. brevicompactum*, as well as all specificity discrepancies between the two machines shown in Table 2, include differences in the temperature ramping dynamics of the instruments, and differences in the DNA polymerases provided in each Master Mix kit. Different DNA polymerases could vary in the effectiveness of their “Hot Start” performance, and in their ability to extend a sequence with a 3' basepair mismatch with an oligonucleotide.

The interference by non-*Aspergillus* species found in this assay is much less than what would be expected from previously published genetic amplification assays, such as the assay presented by Kami *et al.*,¹⁷ which appears to detect the *Aspergillus* genus with the same sensitivity as that of many *Penicillium* species. The “PenAsp” probe presented in Haugland *et al.*¹² is designed to detect both of these genera, and the authors state that sequence alignment indicates that *P. variotii*, *Geosmithia*, *Hemicarpaceales*, and *Monascus* would also be detected.

Future work could include an additional step after qPCR amplification to separate the signal of the “interference” species from that of *Aspergillus*. At basepair 191 of the amplicon, all identified interference species (*i.e.*, *P. variotii*, *P. marneffeii*, *P. glabrum*, and *P. brevicompactum*) have Adenine (A) while all *Aspergillus* analyzed here (*i.e.*, all *Aspergillus* listed in Table 1a) have Guanine (G). This mismatch facilitates unambiguous distinction between amplicons arising from *Aspergillus*, and those arising from other genera identified here as interference species. (Sequences that included this site were considered for probes. However, based on the analysis of NetPrimer, all of the candidate sequences that would be shared by all *Aspergillus* species had a very high probability of dimer formation.)

Although the specificity of the primer/probe set is imperfect, its breadth allows the researcher to detect related species with one qPCR assay. A recent study by Meklin *et al.*³⁶ analyzed house dust from moldy homes and reference homes with qPCR for a variety of fungi. To detect *Aspergillus* species, the study used a total of 24 assays. Although the study found no significant difference in average total concentrations of mold conidia in the dust from moldy and reference homes, there were specific species or groups that did have significantly higher concentrations in the dust from moldy homes. Meklin *et al.*³⁶ suggested it may be possible to evaluate whether a home has an abnormal mold condition by quantifying just six species and groups; four are *Aspergillus*, and another is the *Eurotium* group, which includes *Aspergillus* teleomorphs.³⁶ The use of a single assay such as the one presented here, rather than six, would further simplify the detection of species that may indicate abnormal mold growth.

Results from the sensitivity testing indicated little variation between duplicate qPCR reactions using DNA aliquots from the same extraction and in the same qPCR run (standard deviation between duplicates ranging from 0.01 to 0.16 cycles). This was demonstrated over five orders of magnitude, showing a large dynamic range for the assay.

Variations were seen in the assay's ability to detect a low number of conidia in a sample. In the validation study, the 5th serial dilution point (10^{-5}) of impingers recovering *A. fumigatus* from otherwise pure culture air carried 24 conidia, as calculated by the direct microscopy, and assuming perfect dilution. Although the qPCR results (performed on the MJ Research machine) showed consistent spacing between the first four dilution points, the 5th point was not detected. Juxtaposing results from the pure culture chamber tests with those from the indoor environment (Table 3), several of the air samples were quantified as containing less than 20 conidia. It is possible that the actual quantity in the environmental air samples was >20 conidia, but that inhibition caused an artificially high C_T response. It is also possible that the non-*Aspergillus* particulate matter captured on the filters in the air samples enhanced *Aspergillus* recovery during centrifugation. In contrast, the impinger samples from the laboratory chamber contained only *Aspergillus* conidia, which may not have had the critical mass to form a strong pellet during centrifugation.

The assay amplification efficiency was found to be 82% using laboratory samples of *A. flavus* DNA and 81% using laboratory samples of *A. fumigatus* conidia. These values are within the range recommended by ABI (78%–100%), and their similarity indicates consistent behavior of the assay for samples prepared from laboratory samples of *Aspergillus*. The amplification efficiency was 72% using *A. fumigatus* conidia spiked into sputum—this is slightly lower than the efficiency recommended by ABI, and indicates more inhibition compared with pure culture extractions.

For air sampling, a nominal particle exclusion size ("size cut") corresponding to a mean aerodynamic diameter (mean d_a) of 5 μm was chosen for several reasons. For $\text{PM} > 5 \mu\text{m}$, $>80\%$ of the inhaled particles are removed in the nasal region;³⁷ thus, a PM_5 size-cut focuses on particles more likely to penetrate into the lungs. Also, the design of the cyclone used in these experiments achieved a crisp size exclusion above a mean d_a of 5 μm . Finally, literature indicates that 5 μm is the upper limit for *Aspergillus* particles. Reponen² calculated a range of aerodynamic diameters based on measurements of physical size reported by other research groups for *A. niger*, *A. versicolor*, and *A. fumigatus*, and found them to range between 3.5–5.0, 2.0–3.5, and 2.5–3.0 μm , respectively. The values agree with results from a laboratory study reporting the d_a for *A. versicolor* and *A. fumigatus* conidia to range between 2.0–3.0 μm under different relative humidities;³⁸ furthermore, a field study that collected airborne *Aspergillus* particles in schools of different construction types found the average d_a to fall between 2.33 and 3.25 μm .³⁹

Air sampling results indicated the presence of *Aspergillus* conidia in all fifteen of the indoor environments sampled, with a median concentration of 18 *A. fumigatus* conidia equivalents m^{-3} . Shelton *et al.*¹³ detected *Aspergillus* in 62% of indoor environments across the U.S. using culturing, with a median concentration of 20 CFU m^{-3} in samples testing positive. A number of recent ecological studies have shown that there are large differences between the culture based and non-culture based recovery of bioaerosols,⁴⁰ and our PCR-based observation of *Aspergillus* distribution contributes to the general

finding of this literature. The distribution differences observed with the larger survey conducted by Shelton *et al.*¹³ may be because of the different locations sampled, as well as sensitivity differences between culturing and qPCR. Comparison studies have found qPCR to have higher sensitivity than culturing analysis.^{41,42} One study that quantified four species of *Aspergillus* in house dust with both culturing and qPCR found that standard culture-based methods reported concentrations that were three orders of magnitude lower than qPCR.³⁶ For the purposes of airborne *Aspergillus* particle collection, qPCR should have higher sensitivity. This is in part because it is more inclusive. Culturing detects only viable conidia, and some viable conidia may not grow due to damage during collection or media bias. In contrast, qPCR detects viable and nonviable conidia, as well as hyphal cells; this recovery difference is important when considering the context of hypersensitivity responses to these fungi, since viability is not required to induce a negative health effect. The reported detection limit was 12 CFU m^{-3} for the procedure followed in the culturing study.¹³ For the air samples collected here, 33% had averages less than 12 conidia equivalents m^{-3} , which is a similar value to the 38% of locations found negative in the culturing study.¹³

Underestimation of airborne fungi concentrations by culturing was a major reason for disregarding it as a reference method in this study. Direct microscopic counting was also not used as a reference method for the environmental air samples, because the conidia of *Aspergillus* and *Penicillium* are generally indistinguishable under a light microscope,⁴³ and many common *Penicillium* species are not detected by the assay. However, because air samples were calibrated using pure laboratory cultures of *A. fumigatus*, future work could include estimating the amount of inhibition of the qPCR reaction that is caused by other particulates found in air samples.

For the sputum results, the culturing method used by the clinical laboratory presents data only as positive or negative, so a quantitative comparison between qPCR and culturing results could not be performed. Although only a limited investigation of the assay's ability to diagnose aspergillosis is presented here, the agreement between the culturing and qPCR results shown in Table 4 suggests that the assay has the potential to be used in the clinical setting.

In summary, *Aspergillus* is a mold of health concern that is commonly found in indoor environments. Medical complications caused by exposure to *Aspergillus* range from allergic reactions to fatal pulmonary infections. As the proportion of asthmatics in the U.S. population continues to grow, the need for environmental monitoring to determine the severity and sources of mold such as *Aspergillus*, without inclusion of other molds with strong outdoor sources, also increases. A sensitive, reliable method is critical in the clinical setting as well, where early detection of infection increases the chance for patients' survival, and inclusion of all *Aspergillus* species which can act as opportunistic pathogens is necessary for a thorough screening.

Here, a sensitive and specific qPCR assay that focuses on detection of the *Aspergillus* genus is presented, which has potential for use in environmental research and clinical work.

The entire analysis of both air and clinical samples using the qPCR assay requires less than one work day, so results are quickly obtained. The ability of the assay, in one qPCR reaction, to detect an entire group of related species that are commonly encountered in indoor air, that could potentially be used as indicator organisms for abnormal mold growth, and that includes several species of clinical significance, makes it a simple and financially efficient tool.

Acknowledgements

This work was supported by the Stanford Graduate Fellowship, the Stanford National Institute of Health Graduate Training Program, the Shah Family Fellowship, the Stanford-Singapore Partnership, and the Clorox Company. The authors wish to thank Wing-On Ng, Kai Thormann, Ali Boehm, Ellen Jo Baron, Alfred Spormann, and Mir Nourbakhsh for their advice; the Norman Pace lab at CU-Boulder for equipment usage; Stephen Peterson and Cletus Kurtzman at the USDA-NRRL for providing fungal strains; and Robert L. Goebes for editing assistance.

References

- 1 H. A. Burge, *Ann. Allergy Asthma Immunol.*, 2001, **87**, 52–56.
- 2 T. Reponen, *Aerosol Sci. Technol.*, 1995, **22**, 11–23.
- 3 S. Armstrong and J. Liaw, *ASHRAE J.*, 2002, **44**, 18–24.
- 4 H. A. Burge, D. L. Pierson, T. O. Groves, K. E. Strawn and S. K. Mishra, *Curr. Microbiol.*, 2000, **40**, 10–16.
- 5 W. R. Ott, in *Proceedings of the Research Planning Conference on Human Activity Patterns*, Report No. EPA/600/4-89/004, EPA-NRRL, Las Vegas, NV, 1989, pp. 3-1 to 3-38.
- 6 B. Jacob, B. Ritz, U. Gehring, A. Koch, W. Bischof, H. E. Wichmann and J. Heinrich, *Environ. Health Perspect.*, 2002, **110**, 647–654.
- 7 N. E. Jordanides, E. K. Allan, L. A. McLintock, M. Copland, M. Devaney, K. Stewart, A. N. Parker, P. R. E. Johnson, T. L. Holyoake and B. L. Jones, *Bone Marrow Transplant.*, 2005, **35**, 389–395.
- 8 S. Challier, S. Boyer, E. Abachin and P. Berche, *J. Clin. Microbiol.*, 2004, **42**, 844–846.
- 9 C. E. O'Sullivan, M. Kasai, A. Francesconi, V. Petraitis, R. Petraitiene, A. M. Kelaher, A. A. Sarafandi and T. J. Walsh, *J. Clin. Microbiol.*, 2003, **41**, 5676–5682.
- 10 K. Rantakokko-Jalava, S. Laaksonen, J. Issakainen, J. Vauras, J. Nikoskelainen, M. K. Viljanen and J. Salonen, *J. Clin. Microbiol.*, 2003, **41**, 4304–4311.
- 11 H. Schmidt, M. Bannier, R. F. Vogel and L. Niessen, *Lett. Appl. Microbiol.*, 2004, **38**, 464–469.
- 12 R. A. Haugland, M. Varma, L. J. Wymer and S. J. Vesper, *Syst. Appl. Microbiol.*, 2004, **27**, 198–210.
- 13 B. G. Shelton, K. H. Kirkland, D. W. Flandres and G. K. Morris, *Appl. Environ. Microbiol.*, 2002, **68**, 1743–1753.
- 14 A. L. Pasanen, M. Niininen, P. Kalliokoski, A. Nevalainen and M. J. Jantunen, *Atmos. Environ.*, 1992, **26B**, 121–124.
- 15 N. Mahooti-Brooks, E. Storey, C. Yang, N. J. Simcox, W. Turner and M. Hodgson, *J. Occup. Environ. Hyg.*, 2004, **12**, 826–839.
- 16 Z. Wu, Y. Tsumura, G. Blomquist and X. R. Wang, *Appl. Environ. Microbiol.*, 2003, **69**, 5389–5397.
- 17 M. Kami, T. Fukui, S. Ogawa, Y. Kazuyama, U. Machida, Y. Tanaka, Y. Kanda, T. Kashima, Y. Yamazaki, T. Hamaki, S. Mori, H. Akiyama, Y. Mutou, H. Sakamaki, K. Osumi, S. Kimura and H. Hirai, *Clin. Infect. Dis.*, 2001, **33**, 1504–1512.
- 18 H. Einsele, H. Hebart, G. Roller, J. Loeffler, I. Rothenhofer, C. A. Muller, R. A. Bowden, J. van Burik, D. Engelhard, L. Kanz and U. Schumacher, *J. Clin. Microbiol.*, 1997, **35**, 1353–1360.
- 19 C. A. Rogers, *Immunol. Allergy Clin. North Am.*, 2003, **23**, 501–518.
- 20 J. Loeffler, H. Hebart, R. Bialek, L. Hagemeyer, D. Schmidt, F. P. Serey, M. Hartmann, J. Euker and H. Einsele, *J. Clin. Microbiol.*, 1999, **37**, 1200–1202.
- 21 Z. Wu, X. R. Wang and G. Blomquist, *J. Environ. Monit.*, 2002, **4**, 377–382.
- 22 W. J. Melchers, P. E. Verweij, P. van den Hurk, A. van Belkum, B. E. De Pauw, J. A. Hoogkamp-Korstanje and J. F. Meis, *J. Clin. Microbiol.*, 1994, **32**, 1710–1717.
- 23 J. Peccia, H. M. Perth, S. Miller and M. Hernandez, *Aerosol Sci. Technol.*, 2001, **35**, 728–740.
- 24 W. John and G. Reischl, *J. Air Pollut. Control Assoc.*, 1980, **30**, 872–876.
- 25 J. P. Latge, *Clin. Microbiol. Rev.*, 1999, **12**, 310–350.
- 26 A. R. D. Ganley and T. Kobayashi, *Genome Research*, 2007, **17**, 184–191.
- 27 R. Maleszka and G. D. Clark-Walker, *Yeast*, 1993, **9**, 53–58.
- 28 B. A. Lasker, *J. Clin. Microbiol.*, 2006, **44**, 3145–3153.
- 29 P. C. Y. Woo, K. T. K. Chong, H. Tse, J. J. Cai, C. C. Y. Lau, A. C. Zhou, S. K. P. Lau and K. Y. Yuen, *FEBS Letters*, 2006, **580**, 3409–3416.
- 30 K. Y. Yuen, G. Pascal, S. S. Wong, P. Glaser, P. C. Woo, F. Kunst, J. J. Cai, E. Y. Cheung, C. Medigue and A. Danchin, *Arch. Microbiol.*, 2003, **179**, 339–353.
- 31 N. Vanittanakom, P. Vanittanakom and R. J. Hay, *J. Clin. Microbiol.*, 2002, **40**, 1739–1742.
- 32 L. de Aguirre, S. F. Hurst, J. S. Choi, J. H. Shin, H. P. Hinrikson and C. J. Morrison, *J. Clin. Microbiol.*, 2004, **42**, 3495–3504.
- 33 A. S. Kantarcioglu, G. Hatemi, A. Yucl, G. S. De Hoog and N. M. Mandel, *Mycoses*, 2003, **46**, 45–50.
- 34 A. L. Sunesson, C. A. Nilsson, B. Andersson and G. Blomquist, *Ann. Occup. Hyg.*, 1996, **40**, 397–410.
- 35 C. W. Bayer, S. A. Crow and J. Fischer, *Causes of Indoor Air Quality Problems in Schools*, Report No. ORNL/M-6633/R1, Oak Ridge National Laboratory, U.S. Department of Energy, Oak Ridge, TN, USA, 2000, p.19.
- 36 T. Meklin, R. A. Haugland, T. Reponen, M. Varma, Z. Lummus, D. Bernstein, L. J. Wymer and S. J. Vesper, *J. Environ. Monit.*, 2004, **6**, 615–620.
- 37 J. H. Seinfeld, *Air Pollution: Physical and Chemical Fundamentals*, McGraw-Hill, New York, 1975.
- 38 T. Reponen, K. Willeke, V. Ulevicius, A. Reponen and S. A. Grinshpun, *Atmos. Environ.*, 1996, **30**, 3967–3974.
- 39 T. Meklin, T. Reponen, M. Toivola, V. Koponen, T. Husman, A. Hyvarinen and A. Nevalainen, *Atmos. Environ.*, 2002, **36**, 6031–6039.
- 40 J. Peccia and M. Hernandez, *Atmos. Environ.*, 2006, **40**, 3941–3961.
- 41 M. P. Buttner, P. Cruz-Perez and L. D. Stetzenbach, *Appl. Environ. Microbiol.*, 2001, **67**, 2564–2570.
- 42 C. M. Sedgley, A. C. Nagel, C. E. Shelburne, D. B. Clewell, O. Appelbe and A. Molander, *Arch. Oral Biol.*, 2005, **50**, 575–583.
- 43 D. W. Li and B. Kendrick, *Mycologia*, 1995, **87**, 190–195.