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Development of a method to detect and quantify *Aspergillus fumigatus* conidia by quantitative PCR for environmental air samples

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Abstract

Exposure to *Aspergillus fumigatus* is linked with respiratory diseases such as asthma, invasive aspergillosis, hypersensitivity pneumonitis, and allergic bronchopulmonary aspergillosis. Molecular methods using quantitative PCR (qPCR) offer advantages over culture and optical methods for estimating human exposures to microbiological agents such as fungi. We describe an assay that uses lyticase to digest *A. fumigatus* conidia followed by TaqMan™ qPCR to quantify released DNA. This method will allow analysis of airborne *A. fumigatus* samples collected over extended time periods and provide a more representative assessment of chronic exposure. The method was optimized for environmental samples and incorporates: single tube sample preparation to reduce sample loss, maintain simplicity, and avoid contamination; hot start amplification to reduce non-specific primer/probe annealing; and uracil-*N*-glycosylase to prevent carryover contamination. An *A. fumigatus* internal standard was developed and used to detect PCR inhibitors potentially found in air samples. The assay detected fewer than 10 *A. fumigatus* conidia per qPCR reaction and quantified conidia over a 4–$\log_{10}$ range with high linearity ($R^2 > 0.99$) and low variability among replicate standards (CV = 2.0%) in less than 4 h. The sensitivity and linearity of qPCR for conidia deposited on filters was equivalent to conidia calibration standards. *A. fumigatus* DNA from 8 isolates was consistently quantified using this method, while non-specific DNA from 14 common environmental fungi, including 6 other *Aspergillus* species, was not detected. This method provides a means of analyzing long term air samples collected on filters which may enable investigators to correlate airborne environmental *A. fumigatus* conidia concentrations with adverse health effects.

Key words: airborne fungi, *Aspergillus fumigatus*, conidia, filter, air monitoring, quantitative PCR, sample inhibition

Introduction

Airborne fungi are natural components of the ecosystem and are commonly found in outdoor and indoor environments. Exposure to some species of fungi, primarily via inhalation of asexual spores called conidia, is associated with health problems such as infectious disease, hypersensitivity disease, and toxic effects [1, 2]. *Aspergillus fumigatus* is a common environmental fungus and is the species most frequently associated with a wide spectrum of respiratory disease, including IgE-mediated asthma, invasive aspergillosis, hypersensitivity pneumonitis, and allergic bronchopulmonary aspergillosis [3, 4]. As an opportunistic pathogen, *A. fumigatus* poses a great risk for developing invasive disease in immunocompromised individuals [5]. Invasive aspergillosis (caused by *A. fumigatus* in over 90% of cases) has a limited antifungal therapy success rate of 34% and when

left untreated, has a nearly 100% mortality rate [6, 7]. Hospitals and other environments have been investigated in an attempt to link fungal exposures and adverse health outcomes, but there have been no reports establishing a threshold number of airborne conidia above which there is an increased risk for acquiring a disease such as invasive aspergillosis [8]. Developing accurate, representative exposure assessment methods for pathogenic fungi, such as *A. fumigatus*, is critical for understanding the relationships between exposure, disease, and intervention.

The methods currently used to estimate airborne exposures to fungi have significant limitations and may be a source of disparity in the understanding of the relationship between environmental sampling and health outcomes [9, 10]. Typically, the number and type of airborne fungi are estimated by direct microscopic examination of collected spores or by growing collected spores on an appropriate culture medium under suitable growth conditions [1, 9]. Identification to the species and/or genus level is not always possible with direct examination and short period sampling intervals are necessary to prevent sample overloading which can reduce accurate enumeration [1].

Culture methods also have limitations (Table 1). Some of these limitations necessitate the use of relatively short sample collection times and small collection volumes. A review of publications reporting *A. fumigatus* air sampling in hospital environments showed sampling times were typically less than 20 min in duration and have volumes less than 500 l [11–17]. These brief sample collection periods were then used to characterize exposure periods representing between 2 and 14 days. Since airborne concentrations of fungal conidia can vary by as much as 2–3 orders of magnitude in a 24-hour period [1], it is likely that short period, non-integrated exposure estimates are not representative of actual exposures to airborne conidia. Sampling, using validated filter collection methods, that integrate fungal conidia concentrations over the entire exposure period are clearly needed.

Table 1. Limitations of culture methods for airborne fungi analysis

Harsh conditions during sample collection may render viable fungal spores non-viable
Type of growth media may selectively increase or decrease culture growth
Other microorganisms may inhibit growth of certain fungal types
Desiccation of spores and culture medium during collection significantly limits air sampling time and volume
Non-viable spores in air (which may be allergically significant) do not grow and are not counted
Viable spores may aggregate causing multiple spores to be enumerated as a single colony-forming unit
Quantification is difficult in environments with high spore concentrations due to sampler overloading
Culture collection methods and sampling equipment are not suited for personal monitoring
Limited sample storage time

Molecular biology techniques using PCR have been successfully used for environmental sampling of microorganisms in water, food, soil and air [18–21]. Since PCR does not require that the target microorganism be directly examined or grown on culture medium, sampling time and volume constraints can be eliminated and long term integrated sampling of large volumes of air through filter matrices is possible. This would provide a more representative assessment of chronic exposures which could potentially be used to develop a statistical correlation between the number of conidia in the air and risks associated with developing disease, i.e., an exposure–response relationship [8]. Historically, conventional PCR was only used for detection of nucleic acids and was not quantitative in nature. Recent advances in PCR technology such as TaqMan methods using fluorogenic probes and specialized detectors, allow for real-time quantitative analysis by monitoring the PCR growth curve. Measurement of amplicons present during the early exponential phase of PCR, when reaction components are not rate limiting, provides reproducible and quantitative analysis of nucleic acid. Quantitative PCR (qPCR) methods have been used by several investigators to identify and quantify environmental fungi [19, 22–24].

The published protocols for PCR and/or qPCR of fungi require multiple complex steps designed to breakdown the fungal cell wall, release nucleic acid, and purify/concentrate nucleic acid in a manner suitable for the PCR reaction [5, 19, 22–33]. These steps typically require the transfer of a

sample between multiple tubes, phase separation, and/or filtration. Each of these steps may significantly reduce sensitivity due to loss of nucleic acid, and increase the probability of contamination and variability.

The purpose of this research was to develop a qPCR method for specific detection of *A. fumigatus* conidia deposited on polycarbonate filters using a single tube fungal DNA recovery method suitable for direct addition to the amplification reaction. The method incorporates the use of hot start amplification to reduce non-specific primer annealing, uracil-*N*-glycosylase (UNG) to prevent false positive results due to carry-over contamination, a custom designed internal standard control to identify false negative results due to sample inhibitors, and qPCR product confirmation using fluorogenic probes.

Materials and methods

Fungal genomic DNA preparation

Potato dextrose agar (Becton, Dickson and Company, Sparks, Maryland) plates were inoculated with fungal isolates and incubated for 3–10 days at room temperature. The resultant mycelial mats were removed from the plates using a cell scraper and added to 20 ml of tryptic soy broth and incubated at room temperature for 48 h while shaking at 200 rpm. The liquid culture was then transferred to 50 ml Oakridge polypropylene centrifuge tubes and 2,000 U of lyticase (Lyticase 5,000–20,000 units/mg, Sigma, St. Louis, MO) was added. The mixture was mixed at 5 rpm on a rotating drum for 2 h at room temperature and then incubated without mixing at 37 °C for an additional 2 h. After freezing at −20 °C for 4 h, the solution was heated to 95 °C for 30 min. DNA was recovered using phenol:chloroform extraction and ethanol precipitation according to existing protocols [34] and suspended in 500 μl of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). Serial dilutions of the genomic DNA were then evaluated spectrophotometrically (λ260/280 nm) to determine DNA concentration and purity [34].

A. fumigatus *conidia stock suspension preparation*

Conidia stock suspensions were prepared by harvesting *A. fumigatus* conidia from cultures growing at 25 °C for 6–27 days on potato dextrose agar plates. The conidia were harvested from the plates by gently rolling a dry, sterile, cotton swab over the mycelial mat with as little pressure as possible. The swab was then vigorously swirled in a 15 ml centrifuge tube containing 5 ml of either conidia buffer (deionized ultra pure water [NANOpure® DIamond™ Life Science (UV/UF) ultrapure water system, Barnestread|Thermolyne, Dubuque, IA] containing 0.05% Tween 80) or for comparison purposes using only deionized ultra pure water. A new dry swab was used on an undisturbed area of the plate each time conidia were removed from the surface. After processing four swabs, the tube was centrifuged at 2,000×*g* for 5 min at room temperature. The supernatant was removed and the pellet suspended in 5 ml conidia buffer and briefly vortexed to suspend the pellet. Approximately 80% of the conidia suspension in the centrifuge tube was removed by pipeting from the center of the tube (to avoid removing hyphae and other non-conidial fragments accumulated on the top and bottom layers of the tube) and transferred to a clean 15 ml tube and vigorously vortexed for 60 s. The conidia stock concentrations were then enumerated by hemacytometer (Bright-Line Hemacytometer, Hauser Scientific, Horsham, PA) in accordance with existing protocols [34]. Conidia suspensions were stored at 4 °C for up to 21 days.

Conidial DNA extraction

Dilutions of *A. fumigatus* conidia in conidia buffer were mixed 1:4 with lyticase digestion buffer (5,000 U/ml lyticase, 50 mM Tris, 1.0 μM EDTA, pH 7.5) in a 2.0 ml centrifuge tube and allowed to incubate for 2 h at room temperature with mixing at 180 rpm on a shaker table. After incubation, the tubes were vigorously agitated in a Mini-Beadbeater (Biospec Products, Bartlesville, OK) without beads or other matrices for 30 s at 6,000 oscillations per minute to thoroughly emulsify the sample. An amount of 10 μl of sample was then placed directly into the qPCR master mix. For standard curve preparation, conidial DNA from 50 μl portions of $\log_{10}$ serial dilutions of conidia stock suspensions (original stock solution was enumerated by hemacytometer) were extracted as described above and then analyzed by qPCR. A

new standard curve and corresponding PCR master mix/digestion buffer was made for each sample analysis run.

Filter Processing

Dilutions of stock *A. fumigatus* conidia in conidia buffer (50 μl portions) were placed on the center of 25 mm, 0.8 μm Isopore polycarbonate filters (Millipore Corporation, Bedford, MA) contained in 3-piece, 25 mm polypropylene housings (Millipore Corporation). After drying under a slight vacuum, the filters were placed into 2 ml centrifuge tubes along with 150 μl of digestion buffer (5 U/μl lyticase final concentration) and homogenized at 5,000 oscillations per minute for 30 s using a Mini-Beadbeater (without beads or other matrices) to facilitate removal of conidia from the filter surface. The conidia and conidia digestion buffer were separated from the filter by fixing the filter to the upper portion of the centrifuge tube and centrifuging at 20,600$\times g$ for 5 min. After removing the filter, the conidia pellet and conidia digestion buffer were briefly vortexed and then allowed to incubate for 2-hours at room temperature with mixing at 180 rpm on a shaker table. After incubation, the tubes were vigorously agitated in a Mini-Beadbeater without beads or other matrices for 30 s at 6,000 oscillations per min to thoroughly emulsify the sample. An amount of 10 μl of sample was then placed directly into the qPCR master mix.

Fungal DNA sequence detection

The primers (AF7 and AF8) and probe (AF9) were described by Costa et al. for the multicopy *A. fumigatus* mitochondrial (AfMITO) gene (Genebank accession number: L37095) [23]. The forward primer AF7 (5′-GAA AGG TCA GGT GTT CGA GTC A-3′) and reverse primer AF8 (5′-CAT CAT GAG TGG TCC GCT TTA C-3′) purchased from Invitrogen Custom Primers, Frederick, MD, were used to generate a 196-base pair (bp) amplicon. The fluorogenic probes (AF9 5′FAM-ATC CCT AAA CCC GCA ACC AAA GGC- BHQ-1 3′) and (AFIS probe 5′CAL RED-ACA TCA AAG CTA GCA CCA CCC GCA- BHQ-2 3′) were purchased from Biosearch Technologies Inc., Novato, CA. Initial primer, probe, and amplicon evaluations were confirmed using ethidium bromide staining/gel electrophoresis, Southern transfer with hybridization/colorimetric detection, and DNA sequencing. Universal fungal primers described by Turenne et al. [35] (forward primer, ITS86, 5′-TCC TCC GCT TAT TGA TAT GC-3′ and reverse primer, ITS4, 5′-GTG AAT CAT CGA ATC TTT GAA C-3′) were purchased from Invitrogen Custom Primers, Frederick, MD, and used to confirm the presence of amplifiable, non-*A. fumigatus* DNA during specificity testing experiments using conventional PCR with ethidium bromide staining/gel electrophoresis.

PCR amplification

Quantitative PCR amplification of 10 μl of template was performed in a 25 μl total volume reaction mixture containing 1.5 U AmpliTaq Gold polymerase (Applied Biosystems, Foster City, CA), 1X Gene Amp PCR Buffer II (Applied Biosystems, Foster City, CA), 3.0 μM $MgCl_2$ (Applied Biosystems, Foster City, CA), 0.5 U Amperase uracil-*N*-glycosylase (Applied Biosystems, Foster City, CA), 200 μM dATP, dCTP, dGTP/400 μM dUTP (Promega Corporation, Madison, WI), 0.2 μM AF7 and AF8 primers, and 0.2 μM AF9 probe. PCR amplification and quantitative analysis were performed in a Smart Cycler (Cepheid, Sunnyvale, CA). The qPCR reaction profile used with the Smart Cycler consisted of an initial UNG digestion period (2 min at 50 °C), an AmpliTaq Gold activation interval (10 min at 95 °C), followed by 50 cycles of repeated denaturation (15 s at 95 °C) and annealing/extension (60 s at 65 °C). Threshold concentration (C_t) analysis was performed using Smart Cycler® Software using 2nd derivative curve analysis. The lowest concentration calibration standard which resulted in a C_t value that maintained linearity of the calibration curve and maximized goodness of fit of the regression line was considered the limit of quantification.

Conventional PCR was performed in a 50 μl total volume reaction mixture containing 1.5 U AmpliTaq Gold polymerase, 1$\times$ Gene Amp PCR Buffer II, 3.0 μM $MgCl_2$, 0.5 U Amperase uracil-*N*-glycosylase, 200 μM dATP, dCTP, dGTP/400 μM dUTP, and 0.2 μM ITS 4 and ITS86 primers. A PTC-200 Thermal Cycler (MJ Research, Waltham, MA) was used for initial UNG (2 min at 50 °C) and polymerase (10 min at 95 °C) activation steps which were followed by 40 cycles

of denaturation at 95 °C for 30 s, annealing at 56 °C for 50 s, and extension at 72 °C for 30 s. All experiments included ultra pure water negative controls.

Internal standard control DNA

An internal standard was designed using an 81-bp antisense primer AFpdm (5′-CAT CAT GAG TGG TCC GCT TTA CTA TAT GAA CAC TTT GCG GGT GGT GCT AGC TTT GAT GTT GCA CCT AAA TAT TAT GGT CAT-3′) containing the AFpdm binding sequence located directly upstream from the AF9 probe site, the AFIS probe (a random nucleotide stuffer) sequence, a 13-bp spacer sequence; and the AF8 primer sequence. Using the AFpdm primer in conjunction with the AF7 primer in a PCR reaction with genomic *A. fumigatus* DNA as the template, a 196-bp internal control amplicon containing the sequence corresponding to the AFIS probe in place of the AF9 probe sequence was generated. The resulting internal standard DNA has the same number of base pairs, G:C ratio, and forward/reverse primers as the *A. fumigatus* ''wild-type'' target sequence.

The pCR 2.1-TOPO TA cloning kit (Invitrogen, Carlsbad, CA) was used to clone internal standard amplicons, containing the AFIS probe. An internal standard DNA stock was produced by culturing the clones containing the inserts and extracting plasmid DNA using the QIAfilter Plasmid Maxi Kit (Qiagen, Valencia, CA). The fidelity of the internal standard DNA was confirmed by sequencing. An amount of 1 μl of internal standard DNA, equivalent to 100 PCR units (one PCRU is equivalent to the amount of DNA template in a dilution series which produces detection at endpoint), and 0.2 μM AFIS probe was added into the qPCR master mix to detect sample inhibition.

Particulate matter (PM) was collected for use as a representative PCR inhibitor potentially concentrated during filtration of air. The PM was collected using a high volume cyclone air sampler in urban, ambient Baltimore, Maryland air. Serial dilutions of PM suspended in ultra pure water were added to the qPCR master mix. Samples containing PM suspension were compared to control samples in which PM was not added to the qPCR master mix.

Results

Detection and quantification of A. fumigatus *genomic DNA*

The analytic sensitivity for qPCR using our method was demonstrated over a 7-$\log_{10}$ range (0.0024–2.4×10^4 ng) with replicate analysis of serial dilutions of genomic DNA showing low variability in C_t values (≤ 2.0%) (Table 2). Linear regression analysis between C_t and $\log_{10}$ of *A. fumigatus* DNA concentration per reaction showed the regression line fit the data to a high degree ($R^2 = 1.00$; $y = -3.33x + 42.61$).

There were no false positive results when the AF7/AF8 primer set and AF 9 probe were used in qPCR reactions containing genomic DNA from 14 common environmental fungi, including 6 other *Aspergillus* species (Table 3). The genomic DNA from these non-*A. fumigatus* environmental fungi at 1.0 μg per reaction was not amplified during qPCR. Replicate samples were also analyzed with internal standard DNA (100 PCRU) in the qPCR master mix and did not show any evidence of sample inhibition. As a positive control, each environmental fungus isolate shown in Table 3 was also evaluated using conventional PCR with universal primers. All isolates (1.0 μg per reaction) produced a discrete band upon gel electrophoresis/ethidium bromide staining confirming the presence of fungal DNA (Table 3). Additionally, conidial DNA (approximately 10–150 conidia per reaction) from 8 different *A. fumigatus* isolates were evalu-

Table 2. qPCR analytical sensitivity of *A. fumigatus* (ATCC 42203) genomic DNA

DNA[a] (ng)	# No. of Replicates	Average C_t (coefficient of variation (%)
2.4×10^4	6	14.3 (2.0)
2.4×10^2	6	21.3 (1.6)
2.4	6	28.4 (0.5)
0.24	6	31.7 (0.7)
0.024	6	34.5 (1.8)
0.0024	5	37.2 (1.7)[b]

[a] As measured by spectrophotometric analysis of *A. fumigatus* DNA at 260 nm.

[b] Calculations based only on replicates with growth curve above threshold (1 of 6 replicates did not have growth curve above threshold).

Table 3. Summary of specificity testing results

Fungal species[a]	AFU specific primers (AF7/8) and probe[b] (AF9)	Universal primers[c] (ITS4/86)	Internal standard primers (AF7/8) and probe[d] (AFIS)
A. fumigatus (ATCC 42203)[e]	+	+	+
A. fumigatus (JHU 01) [e]	+	+	+
A. fumigatus (JHU 02) [e]	+	+	+
A. fumigatus (JHU 03) [e]	+	+	+
A. fumigatus (JHU 04) [e]	+	+	+
A. fumigatus (JHU 05) [e]	+	+	+
A. fumigatus (JHU 06) [e]	+	+	+
A. fumigatus (JHU 07) [e]	+	+	+
A. flavus[f]	–	+	+
A. glaucus[f]	–	+	+
A. niger[f]	–	+	+
A. terreus[f]	–	+	+
A. ustus[f]	–	+	+
A. versicolor[f]	–	+	+
Alternaria sp. [f]	–	+	+
Bipolaris sp. [f]	–	+	+
Cladosporium sp. [f]	–	+	+
Curvularia sp. [f]	–	+	+
Fusarium sp. [f]	–	+	+
Paecilomyces lilacinus[f]	–	+	+
Penicillium sp. [f]	–	+	+
Rhizopus sp. [f]	–	+	+

[a] All isolates, with the exception of *A. fumigatus Fresenius*, were identified and provided by the Johns Hopkins Hospital Clinical Mycology Laboratory.
[b] qPCR using AF 7/8 primer and AF9 probe (+: equals amplification; –: equals no amplification).
[c] Conventional PCR with ethidium bromide gel electropheresis using ITS 4/86 primers (+: equals amplification; –: equals no amplification).
[d] qPCR using AF 7/8 primer, AFIS probe, 100 PCRU internal standard DNA (+: equals no inhibition detected; –: equals inhibition detected).
[e] Fungal conidial DNA equivalent to approximately 10–150 conidia per amplification reaction.
[f] 1.0 μg purified fungal DNA per amplification reaction.

ated, as described above, and no false negative results were observed (Table 3).

Detection and quantification of A. fumigatus *conidia*

Hemacytometer count variability and the frequency of conidia clumping (occurrence of two or more conidia in physical contact) were much greater when deionized water was used in place of conidia buffer during harvesting. The coefficients of variation for replicate hemacytometer counts (three replicate counts performed per dilution with approximately 180–240 conidia per counting grid) of five different conidia stocks suspended in conidia buffer ranged from 9.4% to 14.9%. Corresponding hemacytometer counts (three replicate counts per dilution with approximately 150–200 conidia per counting grid) for conidial dilutions made from five stocks of conidia suspended in deionized water ranged from 12.6% to 64.6%. An average of 4.5% (S.D. = 0.3%; $n = 6$ replicate counts per dilution) of conidia counted were clumped together when suspended in conidia buffer (approximately 180–240 conidia per counting grid), while an average of 36% (S.D. = 9.2%; $n = 6$ replicate counts per dilution) of conidia counted were clumped together when suspended in water (approximately 100–180 conidia per counting grid). The coefficient of variation for hemacytometer counts for five replicate dilutions of conidia suspended in conidia buffer (177–200 conidia per counting grid) was 6.0% and the coefficient of variation for hemacytomer counts for five replicate counts of the same dilution of conidia suspended in conidia buffer was 5.4%. The amount of growth time (between 6 and 27 days) before harvesting did not have any discernable effect on qPCR or hemacytometry (data not shown). Storage of conidia stocks at 4 °C for up to 21 days did not have an effect on qPCR results when compared to immediate qPCR analysis (data not shown).

To evaluate qPCR variability associated with analysis of *A. fumigatus*, 50 μl portions of $\log_{10}$ serial dilutions of conidia stock (original stock solution was enumerated by hemacytometer) ranging in concentrations from approximately 10 to 10^5 conidia per ml were digested in lyticase and analyzed by qPCR. An analysis of replicate digestions of a 5-$\log_{10}$ range of serial dilutions of *A. fumigatus* conidia stock suspensions (Table 4) showed low variability among replicates (≤ 1.5%) and the goodness of fit of the regression line ($R^2 = 0.99$; $y = -3.58x + 42.7$) was maintained.

We consistently and repeatedly noted a difference in C_t values for filter samples and matched no filter controls in our experiments. The C_t values for conidia digests were on average 2.9 cycles lower when filters were present during processing compared with control samples which did not have filters present during processing ($n = 14$ filters evaluated, mean = 827 conidia/qPCR reaction,

Table 4. Sensitivity of qPCR for detection of *A. fumigatus* (ATCC 42203) conidia after lyticase digestion

# No. of conidia per PCR reaction	# No. of replicates	Average C_t (coefficient of variation (%)
60625	3	25.8 (1.3)
6063	3	29.2 (0.5)
606	3	32.2 (0.8)
61	3	36.7 (1.5)
6	3	38.0 (2.4)[a]
0.6	3	37.8 (3.6)[a,b]

[a] Values not included in regression equation for determination of standard curve slope and fit. Corresponding C_t values in this range for unknown samples are considered detectable, but not quantifiable.
[b] Calculations based only on replicates with growth curve above threshold (1 of 3 replicates did not have growth curve above threshold).

range = 25–1700 conidia/qPCR reaction). These differences were statistically significantly different ($P = 0.001$) based on a *t*-test with a 95% confidence interval.

Standard curves produced during our investigations using seeded filters (Table 5) showed a mean limit of quantification of 6 conidia per 10 μl reaction and mean R^2 value of 0.99. Standard curves produced using conidia dilutions without filters present during sample processing had a mean limit of quantification of 11 conidia per qPCR reaction with a mean R^2 value of 0.99 (Table 5). The mean slope of the standard curves for filter samples and no-filter samples were similar (Figure 1a), while a comparison of intercept values shows an offset between the two groups of curves (Figure 1b).

Evaluation of internal standard DNA

The C_t values for reactions containing 100 PCRU of internal standard DNA and ultra pure water ($n = 15$) had a standard error of 0.06. The addition of 120 μg of PM suspension per reaction completely inhibited amplification of both 100 PCRU of internal standard and the DNA equivalent of 50 *A. fumigatus* conidia. When the PM suspension concentration was decreased to 12 μg per reaction the internal standard DNA continued to be completely inhibited, while the C_t value for conidial DNA increased by 1.8 cycles (indicating partial inhibition) when compared to control samples without PM suspension. A further $\log_{10}$ decrease in PM suspension concentration (1.2 μg per reaction) did not markedly alter the C_t values (< 0.5 cycles) for either the internal standard DNA or conidia DNA when compared to controls.

The addition of internal standard DNA, equivalent to 10 PCRU, to qPCR reactions containing 2.4 pg of *A. fumigatus* DNA resulted in a change of 1.9 cycles for *A. fumigatus* C_t. There were no marked changes (< 0.5 cycles) for 10 PCRU of internal standard DNA when concentrations of *A. fumigatus* DNA were increased to 24 pg. When 100 PCRU of internal standard DNA was added to 2.4 pg of *A. fumigatus* DNA there was no amplification of *A. fumigatus* DNA. When 100 PCRU of internal standard DNA was added to 24 pg *A. fumigatus* DNA there was a change of 2.5 cycles. There were no changes in C_t more than 0.5 cycles when either 10 or 100 PCRU of internal standard DNA was added to 240 pg of *A. fumigatus* DNA.

Discussion

Our study is unique in that we have optimized qPCR analysis for specific detection of *A. fumigatus* conidia from environmental samples. Costa et al. developed one of the first qPCR methods for the detection of *A. fumigatus* DNA in clinical serum samples [23]. However, their study did not focus on conidia nor address concerns associated

Table 5. Summary of standard curves generated from qPCR analysis of $\log_{10}$ dilutions of *A. fumigatus* (ATCC 42203) conidia after lyticase digestions protocol

	N	Limit of Quantification[a] mean (range)	Slope mean (range)	Intercept mean (range)	R^2 mean (range)
No filters	10	11 (2–47)	-3.3 (-2.7 to −3.6)	39.7 (37.8–41.5)	0.99 (0.98–1.0)
Filters	9	6 (1–20)	-3.3 (-2.8 to −3.7)	35.9 (33.0–38.4)	0.99 (0.95–1.0)

N is the number of standard curves analyzed. Each curve contains at least four data points and span greater than 3-$\log_{10}$ orders of magnitude above the limit of quantification.
[a] Conidia/qPCR reaction.

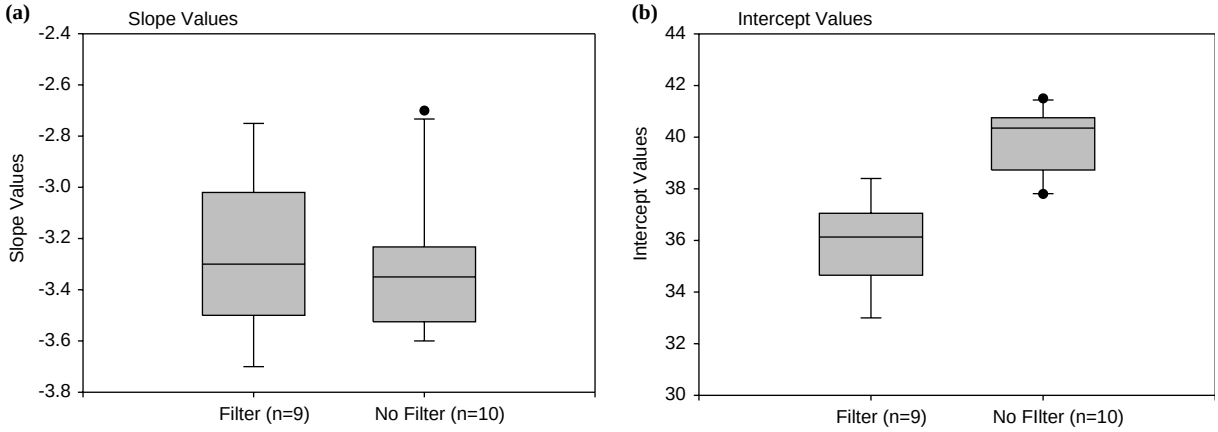

Figure 1. Box plots showing distribution of slope (a) and intercept (b) values for *A. fumigatus* conidia standard curves processed either with or without filters present. The bottom and top box edges represent the 25th and 75th percentiles of the distribution, the horizontal line through the box represents the median, the central vertical lines extending from the box represent the range of data falling within the 10th and 90th percentiles, and dots represent outliers.

with environmental sampling such as DNA release from conidia, use of internal controls, or specificity with respect to environmental fungi. Their characterization of the multicopy mitochondrial gene (9–10 mitochondrial targets per one single copy gene) did provide a means to increase qPCR signal and more reliably detect low numbers of conidia. This is especially important for the analysis of environmental samples in which detection of very low concentrations of target is typically necessary.

We have achieved qPCR performance similar to Costa et al. [23] using the same primers and probes. The sensitivity, variability and range of detection are consistent with those results reported by other investigators using qPCR to detect *A. fumigatus* conidia [5, 36]. Our values are also consistent with those methods developed for the detection of *S. chartarum* spores with respect to sensitivity and range [19, 22, 24]. We have also shown that the primers and probes used with this method were specific for *A. fumigatus* and will not detect DNA from other *Aspergillus* species or non-*Aspergillus* species of fungi commonly found in the environment (Table 3).

Fungal cell wall degradation is crucial for releasing nucleic acid needed for qPCR analysis. Many methods including glass bead milling, enzymatic digestion, freeze/thaw, sonication, and other physical disruption methods have been used to breakdown the fungal cell wall for this purpose [5, 19, 22–33]. Many of these methods are complex, requiring concentration steps, clean-up steps, and specialized equipment. Enzymatic methods, using enzymes such as lyticase, may be the method of choice to consistently release DNA from fungi and have been previously used for this purpose [29]. Lyticase degrades fungal cell walls by cleaving the $\beta(1–3)$ glycosidic bonds between glucose moieties resulting in the breakdown of the rigid, water insoluble skeletal portion of the cell wall [38, 39]. The concentrations of lyticase buffer used in this study did not cause inhibition of the PCR reaction and effectively released DNA. Enzymatic digestion using lyticase facilitates the use of a single tube, reduces sample handling, and is sensitive enough to detect less than one conidium. Threshold cycle values corresponding to less than one conidium are possible since detection is based on the multicopy mitochondrial gene.

With TaqMan analysis the threshold cycle (C_t) is the fractional PCR cycle at which there is a significant increase in fluorescent signal above a defined threshold. The relationship between the log of initial target copy number in a set of standards and the C_t values for the respective standards is linear. This standard curve is used to convert the C_t values from test samples into copy numbers of template. At low template concentrations near detection endpoint, C_t values tend to vary from the log linear relationship and cause a decrease in the goodness-of-fit (R^2) of the calculated regression line. For this reason, it is sometimes necessary to exclude these non-linear endpoint values from the regression calculation

and accept a higher limit of quantification. In these cases, samples with a C_t near the endpoint may be considered positive, but the quantity of nucleic acid cannot be predicted with the regression equation.

The presence of inhibitors and the need for internal standards in qPCR has been identified previously [5, 24]. When incorporated into qPCR analysis of inhibitor-containing PM (e.g., collected from urban air) samples, our internal standard DNA was completely inhibited at PM concentrations that resulted in partial or complete inhibition of *A. fumigatus* conidial DNA. Thus, addition of internal standard DNA to unknown samples can effectively show when inhibitors, such as those present in the air, are present in a sample. In cases where the internal standard C_t value for a sample is significantly different from an established reference value or is completely inhibited, the corresponding C_t value for the *A. fumigatus* DNA cannot be quantified with certainty. Theoretically, multiplex analysis with detection of internal standard DNA and *A. fumigatus* DNA in the same reaction tube can be used to detect inhibitors. However, we have noted interaction between the internal standard and target DNA which results in unreliable C_t values. The interaction effect was present near endpoint concentrations of *A. fumigatus* DNA even when only low amounts (10 PCRU) of internal standard DNA were present. Increasing internal standard concentrations resulted in greater interaction effects. We hypothesize that the differential inhibition is due to the exhaustion of rate limiting components contained in the qPCR master mix. It is possible that when low concentrations of native template DNA are present in samples, the growth curve for internal standard DNA occurs at an earlier cycle and begins to reach its plateau (indicating reaction components have become rate limiting). As a result, essential components for amplification of native DNA are not available and a 'normal' growth curve is not possible. To maximize sensitivity and eliminate interaction between target and internal standard, use of a separate tube for detection of inhibitors is recommended.

Accurate development, maintenance, and enumeration of conidial stock suspensions were critical because these stocks provided the basis for developing standard curves. Variability in estimating the concentrations of stock suspensions will be reflected in variability in determinations of unknown samples. The use of a surfactant such as Tween 80 was essential for making accurate serial dilutions. Without Tween 80, serial dilutions were inconsistent and contributed to lower R^2 values noted for standard curves made using deionized ultra pure water conidia stocks compared to standard curves made from conidia buffer stocks (data not shown).

Use of polycarbonate filters was compatible with our qPCR method. The sensitivity and linearity of qPCR for conidia seeded onto filters was equivalent to conidia calibration standards. Interestingly, a comparison of the qPCR analyses of conidia seeded onto filters and corresponding no filter controls show lower C_t values (more signal) when filters are present during digestion (Table 5 and Figure 1b). The average difference is about 3 C_t units which is equivalent to a log difference in conidia number. Based on experiments done in our laboratory (data not shown), we believe the increased signal associated with filters is due to increased mechanical forces created by the filter in the centrifuge tube during homogenization. To prevent making a biased standard curve we recommend placing a clean/unused filter in the centrifuge tube along with conidia calibration standards to account for the enhanced qPCR signal expected with sample processing.

This assay has sensitivity that would allow it to be used with environmental sampling. The analytical limit of quantification for the qPCR method is approximately 6 conidia per PCR reaction or 90 conidia per filter (assuming a 10 μl qPCR sample volume per qPCR reaction and a 150 μl digestion volume). For an air sample collected over an 8 h period at a collection rate of 15 l per minute, the collection and analysis limit of quantification would be 13 conidia/m^3 of air. Thus, the qPCR filter collection and analysis method developed would have equivalent or better sensitivity than other commonly used sampling methods (such as the Anderson N-6 impactor or AGI-30 impinger) used to quantify airborne concentrations of *A. fumigatus* conidia. Although, a single colony forming unit can be enumerated with either of these other collection and analysis methods, constraints due to limited volumes of air collected (Anderson N-6) or from dilution in liquid collection media (AGI-30) lowers the overall sensitivity of these collection and analysis methods. Since filter based methods are

not limited to short sampling periods, larger volumes of air may be collected, thereby, reducing the overall collection and analysis limit of quantification for airborne conidia.

We have developed a rapid and reliable qPCR method specific for quantitative detection of *A. fumigatus* conidia. The method incorporates hot start amplification to reduce non-specific primer annealing, uracil-*N*-glycosylase to prevent false positive results due to cross-over contamination, a custom designed internal standard to identify false-negative results due to PCR inhibitors, use of primers for multi-copy genes to increase detection sensitivity, and the use of second derivative analysis to reduce variability in C_t determination due to threshold differences. This method uses lyticase digestion to facilitate single tube sample processing to minimize variability and maximize sensitivity. Our investigations demonstrate that TaqMan™ qPCR can specifically detect low numbers of fungi (at the species level) and produce a highly linear standard curve in less than 4 h. The use of this molecular qPCR method will provide a means of analyzing longer term air samples and enable investigators to correlate exposure to airborne fungi with adverse health effects.

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