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Use of Green Fluorescent Protein-Expressing *Aspergillus fumigatus* conidia to Validate Quantitative PCR Analysis of Air Samples Collected on Filters

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This study used green fluorescent protein (GFP)-expressing Aspergillus fumigatus *conidia to compare quantitative PCR (qPCR) enumeration with direct epifluorescent microscopic filter counts of conidia collected on filters in a test chamber. In separate experiments this study initially compared white versus fluorescent light microscopy for counting* A. fumigatus *conidia, then compared fluorescent microscopy counting of corresponding filter halves, and finally compared qPCR enumeration to counting by fluorescent light microscopy. The use of GFP-expressing conidia with epifluorescent microscopy yielded significantly higher conidia counts ($p = 0.026$, $n = 41$, mean of 4.1 conidia per counting field) and 40% faster counting times when compared to conventional counting using white light microscopy. GFP-expressing conidia were aerosolized in a test chamber and collected onto filters. Filters were divided in half and GFP-expressing conidia enumerated. There was no significant difference in the average conidia count per field between corresponding filter halves ($p = 0.3$, $n = 9$ filters, mean of 7.8 conidia per counting field). Thus, one filter half could be counted optically and would provide a reliable estimate of filter loading of the corresponding half, which could then be analyzed by qPCR. Filters ($n = 38$) loaded with GFP conidia in the aerosol chamber were divided in half and analyzed by either fluorescent microscopy or qPCR. The estimated filter loadings ranged from 15–30,000 conidia per filter. There was a linear relationship with a nearly 1:1 ratio between qPCR and direct microscopic estimates of filter loading ($y = 1.06\times + 404$; $R^2 = 0.91$) showing that the outlined qPCR analysis method is in agreement with an external reference method and is reliable for enumerating* A. fumigatus *conidia collected on filters. The comparative data derived using GFP-expressing conidia confirmed that qPCR provides sensitive and accurate quantification of DNA from airborne conidia collected on filters.*

Keywords airborne fungi, *Aspergillus fumigatus*, filter, green fluorescent protein, monitoring, quantitative PCR

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Exposure to airborne fungi has been associated with a myriad of human health effects ranging from localized respiratory irritation to invasive diseases with high mortality rates.[1–3] Health effects associated with airborne fungal exposures are a function of the type of fungi and dose of inoculum.[4,5] Therefore, exposure assessment methods used to determine risk of disease, association between exposure and disease, or efficacy of environmental intervention methods must be able to provide representative data on the species and number of fungi present. The asexual reproductive propagates of many environmental fungi, called conidia, are easily aerosolized and capable of surviving in the air for extended periods of time (note that although the term "spore" is often used to describe asexual fungal propagates, this usage is incorrect and should be applied only to sexually formed propagates).[1] For the purposes of this study we selected the fungal species *Aspergillus fumigatus* as a representative fungal microorganism. *A. fumigatus* is commonly found in indoor and outdoor air and is the fungal species most frequently associated with a wide spectrum of respiratory disease, posing a high mortality risk for immunocompromised persons when invasive infection occurs.[6]

Assessing exposure to airborne fungi, such as *A. fumigatus*, is problematic since reliable and representative environmental air sampling methods are lacking.[6,7] Traditionally, airborne fungi exposures have been determined by culture analysis or by direct microscopic evaluation. Sample collection methods for culture analysis can directly affect the survival of culturable fungi and underestimate airborne concentrations, thereby reducing sampling accuracy.[8,9] Due to desiccation of media and organisms, as well as media overloading, air sampling is often limited in time and air volume.[1] Optical methods used to enumerate fungi have limited capacity to identify organisms to the species level, are subject to media overloading, and often require very lengthy analysis by a highly trained individual.[1] Continuous recording devices that impact spores onto moving

glass slides or tapes can be analyzed optically and used in conjunction with culture methods to obtain integrated sample results to species level, but they require large amounts of time be spent in the field and require special sample handling. These limitations usually preclude the ability to easily perform integrated sampling throughout the day and bring the representativeness of the resulting exposure estimates into question. Easy to use sampling methods that require minimal time spent in the field and integrate concentrations of airborne fungi over the entire human exposure period are clearly needed to allow investigators to accurately assess human exposures in an efficient manner.

The use of molecular analysis offers a potentially robust means to evaluate environmental samples. Molecular biology techniques using PCR have been used successfully for environmental sampling in water, food, soil, and air for fungi and other pathogenic organisms.[10–13] Since PCR does not require the collected sample to be grown on culture medium, most sampling limitations associated with growth in culture are negated. Similarly, problems associated with sample desiccation and overloading are not relevant. Because PCR typically has a linear response over many orders of magnitude, analysis of spores on filters can be performed over a wide range of filter loading. In addition, since PCR response is a function of filter loading, and not environmental concentration, sampling time and volume constraints are eliminated making long-term integrated sampling of large volumes of air (with corresponding increase in the sensitivity and representativeness of the sample) possible.

A recent advance in PCR technology is the development of quantitative nucleic acid detection methods. Methods using quantitative PCR (qPCR) have been proposed as an improvement over culture and microscopic methods traditionally used to enumerate fungal air samples for the purpose of exposure assessment.[13] In qPCR analysis, a fluorogenic DNA probe is used to directly monitor amplification of nucleic acid during PCR thermocycling. The cycle threshold at which the fluorescent signal is detected over background is used to calculate the initial amount of DNA present in a sample. qPCR standard curves generated using known amounts of DNA enable the quantification of DNA in unknown samples. The ability to collect time-integrated samples will provide a more representative assessment of chronic exposure offering a clear improvement over short-term samples currently cited in the literature.

Collection of particulate matter on filters with subsequent laboratory analysis is one of the preferred methods used to evaluate aerosols for exposure assessment.[14] Filters vary with respect to material, pore size, pore type, nominal sampling flow rate, reactivity, and hydrophilicity or hydrophobicity.[15–17] Some of these characteristics will favor air sample collection and particle retention, while others may favor conidia extraction from the filter. The use of cellulose ester, polycarbonate, and fluoramide filters has been cited as filter media suitable to collect bioaerosols.[18–20] Membrane filters housed in polypropylene cassettes have been shown to have 100% collection efficiency for particles up to 20 μm aerodynamic equivalent diameter (well within the size range of many fungal conidia), and filters are amenable to sampling over long periods of time and for drawing large volumes of air.[8] They can be used with high-volume pumps for stationary area sampling or with lightweight, battery-powered pumps for personal sampling. Thus, use of filters for sample collection offers an advantage over many commonly used culture sample collection methods that are not well characterized or convenient for field use.[21]

The use of qPCR to analyze air samples collected on matrices, such as filters, has been proposed by commercial laboratories and by the U.S. Environmental Protection Agency.[22] However, qPCR sample processing and analysis protocols have not been adequately validated in scientific peer-reviewed literature. Ideally, qPCR analysis of air samples would be validated against an external reference value or "gold standard" to ensure PCR analysis methods are providing an accurate estimate of fungal loading. However, there are currently no gold standards in existence to use as reference.

Comparison of qPCR collection and analysis methods to existing culture or conventional microscopic methods is inadequate. Culture methods only enumerate culturable conidia, whereas qPCR is able to enumerate total conidial DNA when suitable primers and probes are available for analysis. Microscopic counting of conidia on filters provides a direct means of estimating filter loading, but is time consuming, technically challenging, and inaccurate due to the lack of contrast between translucent conidia and the filter surface.

The green fluorescent protein (GFP) of the *Aequorea victoria* jellyfish is an autofluorescing protein that has been used extensively as a unique tool to label individual cells and whole organisms.[23] GFP tagged microorganisms can be monitored or enumerated in real time by fluorescence microscopy. The previous development of a GFP-expressing *A. fumigatus* construct[24] provides the opportunity to compare qPCR enumeration with direct epifluorescent microscopic filter counts of airborne GFP-expressing *A. fumigatus* conidia, collected on filters within a test chamber.

METHODS

A. fumigatus conidia Stock Suspension Preparation

Hygromycin-resistant gGFP *A. fumigatus*[24] was inoculated onto potato dextrose agar plates containing 250 μg/mL hygromycin (Sigma, St. Louis, Mo.) and incubated at room temperature for 7–10 days. *A. fumigatus* conidia were harvested into conidia buffer (ultrapure water containing 0.0005% Tween 80) as described previously.[25] Briefly, conidia were harvested by gently rolling a cotton swab over the surface of the mycelial matt, vigorously swirling the swab in a 15-mL centrifuge tube containing conidia buffer, and repeatedly rinsing and pelletizing using centrifugation (500 $\times$ g for 5 min). Conidia stocks were enumerated with a hemacytometer and concentrations were adjusted to approximately 10^7 conidia per mL.

Fluorescent Microscopy Analysis

GFP-expressing *A. fumigatus* conidia were dry mounted onto glass slides to evaluate the percentage of conidia expressing GFP. Conidia were viewed at 400× total power using an Olympus B60 microscope (Olympus America Inc., Lake Success, N.Y.) with blue light and fluorescein filters. Digital photographic analysis was used to overlay fluorescent and white light fields of view.

Aerosol Generation and Sample Collection

Aerosols of *A. fumigatus* were generated in a sealed 58-L (44 × 45 × 29.3 cm) polycarbonate chamber equipped with a mixing fan. The chamber was purged with HEPA filtered air for at least 60 min before and after aerosol generation. The chamber was supplied with aerosol via a 3-jet Collison Nebulizer (BGI Incorporated, Waltham, Mass.) containing a 45 mL suspension of gGFP-expressing *A. fumigatus* conidia (approximately 10^7 conidia per mL of conidia buffer). The nebulizer pressure was adjusted to between 69 and 138 kPa to provide the desired concentration of conidia in the chamber. Particle concentrations within the chamber were monitored in real-time using a TSI 3320 Aerodynamic Particle Sizer (TSI Inc., Shoreview, Minn.) for the purpose of determining sampling times and flow rates needed to yield appropriate filter loadings.

Samples were collected using a 0.8-μm, 25-mm Isopore polycarbonate filter supported by a 25-mm cellulose support pad (Millipore Corporation, Bedford, Mass.) contained in a three-piece, 25-mm polyproplylene cassette. Air was drawn through the open-face filter cassette at 2–3 L/min via an Aircheck Sampler pump (SKC Inc., Eighty Four, Pa.). The pump was calibrated before and after sampling using a Dry Cal DC-2 Flow Calibrator (Bios International Corp., Butler, N.J.). Sample collection times were varied from between 5 and 15 min based on the chamber aerosol concentration and the desired filter loading densities.

Filter Processing

Post-Sampling Preparation

After sample collection in the aerosol chamber, each filter was removed from the filter cassette using flame-sterilized forceps and placed on a cutting template. The template consisted of a clean glass slide that was permanently marked with a 25-mm square containing diagonal lines between adjacent corners. The filter was aligned inside of the square and cut in half along one of the diagonal lines with a flame-sterilized #20 scalpel using a rocking motion. One-half of the filter was removed from the template and mounted on a 25-mm × 75-mm glass slide using a 22-mm × 40-mm No. 1 glass cover slip attached with clear nail polish and stored away from light until analyzed by epifluorescence microscopy. The other filter half was placed into a 2.0 mL microcentrifuge tube and stored at 4°C until qPCR analysis. Initial experiments (n = 9) consisted of mounting both filter halves on separate glass slides for the purpose of evaluating counting reproducibility.

Chamber Filter Microscopy Analysis

Filter halves loaded with conidia in the generation chamber experiments were viewed at 400× total power using an Olympus B60 microscope with blue light and fluorescein filters. All conidia viewed within a 0.0625 mm^2 graticule, at multiple field depths were counted. The slide was viewed in a standard pattern to ensure that representative areas of the entire filter surface were evaluated. The filter was bisected and the stage was moved in successive linear paths perpendicular to the line of bisection. Graticule fields were selected randomly by looking away from the eyepiece briefly while moving the mechanical stage. Counting was stopped after either a minimum of 100 conidia were counted or 100 fields were viewed.

As filter loadings increased, the distances between graticule fields and perpendicular traces were increased to ensure that a representative sample of the entire surface was included in the count. The number of fields observed and conidia counted were noted for each filter and used to determine the average number of conidia per area of filter viewed. This number was then multiplied by the filter area to determine the total number of conidia per filter half. Blank filters were included as negative controls. Slide-counting order was randomized and performed without knowledge of qPCR results for the corresponding filter half to eliminate potential counting bias.

Chamber Filter qPCR Analysis

For qPCR analysis, filters were processed and analyzed as described previously.[25] Briefly, the filter half was placed into a 1.5-mL centrifuge tube containing 5-μL lyticase buffer (40 U/μL lyticase, 50 mM Tris, 1.0 μM EDTA, pH 7.5) and 145 μL ultrapure water and agitated in a mini-beadbeater (the beadbeater was used without beads as a mixer) at 5000 rpm for 30 sec. After agitation, the filter was fixed to the upper side of the centrifuge tube by wedging the top edge of filter between the centrifuge tube wall and the inner wall of the tube lid and spun dry at 23,000 × g for 5 min. After removing and discarding the filter, the conidia digest was incubated for 60 min at 25°C, then agitated with the mini-beadbeater for 30 sec at 5000 rpm and stored at 4°C until qPCR analysis.

TaqMan qPCR was performed using the forward primer (AF7) 5′-GAA AGG TCA GGT GTT CGA GTC A-3′, reverse primer (AF8) 5′-CAT CAT GAG TGG TCC GCT TTA C-3′, and probe (AF9) 5′FAM-ATC CCT AAA CCC GCA ACC AAA GGC- BHQ-1 3′ described by Costa et al.[26] for the multicopy *A. fumigatus* mitochondrial gene (Genebank accession number: L37095). Primers were purchased from Invitrogen Custom Primers (Frederick, Md.) and probes were purchased from Biosearch Technologies, Inc. (Novato, Calif.). The qPCR amplification of 10 μL of conidia digest was performed in a 25 μL total volume reaction mixture containing 1.5 U AmpliTaq Gold polymerase (Applied Biosystems, Foster City, Calif.), 1X Gene Amp PCR Buffer II (Applied Biosystems, Foster City, Calif.), 3.0 μM $MgCl_2$ (Applied Biosystems) 0.5 U Amperase Uracil N-glycosylase (UNG) (Applied Biosystems), 200 μM dATP, dCTP, dGTP/400 μM dUTP (Promega

Corporation, Madison, Wisc.), 0.2 μM AF7 and AF8 primers, and 0.2 μM AF9 probe. UNG is an enzyme used in molecular biology to prevent carryover contamination from existing PCR amplicons into samples by degrading uracil-containing PCR products prior to amplification. Briefly, deoxythymidine-triphosphate is replaced with uracil containing deoxyuridine-triphosphate during PCR amplification. As a result, if amplified DNA sequences from previous experiments (which now contain uracil in the DNA sequence) happen to contaminate a new sample during processing, these amplicons are destroyed by UNG prior to amplification.

PCR amplification and quantitative analysis were performed in a SmartCycler (Cepheid, Sunnyvale, Calif.) and 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 sec at 95°C) and annealing/extension (60 sec at 65°C).

Threshold concentration (C_t) analysis was performed using SmartCycler Software 2.0 using second derivative curve analysis. For standard curve preparation, conidia from 50 μL portions of $\log_{10}$ serial dilutions of stock suspensions enumerated by hemacytometer (Bright-Line Hemacytometer; Hauser Scientific, Horsham, Pa.) according to the manufacturer's instructions, were mixed with 5-μL lyticase digestion buffer, 95-μL ultrapure water, and a clean filter half and were prepared and digested as described above, followed by qPCR analysis. A new standard curve was made for each sample analysis run. A single PCR master mix was prepared for each group of standards and samples. Blank filters and blank reagent controls were included with each sampling and analysis run.

Data Analysis

Statistical analysis was performed using Excel 2002 (Microsoft Corporation, Redmond, Wash.) for t-tests and scatter plots, Intercooled Stata 6.0 (Stata Corporation, College Station, Texas) for regression analysis, and Sigma Plot 8.0 (Systat Software Inc., Point Richmond, Calif.) for box-plot analysis. Standard curves were generated using simple linear regression where cycle threshold was the dependent variable and the $\log_{10}$ number of *A. fumigatus* conidia was the independent variable. Comparison of conidia counts using fluorescent microscopy (dependent variable) and qPCR analysis (independent variable) were performed using simple linear regression. Confidence limits for all tests were set at 95%.

RESULTS

When GFP-expressing *A. fumigatus* conidia were dry mounted on a glass slide (no filter present) the conidia were clearly visible and easily enumerated under white light and fluorescence microscopy. Digital photographic analysis used to overlay fluorescent and white light fields of view displayed 100% agreement between white light and fluorescent conidia counts (n = 106 conidia compared) indicating that all conidia were expressing GFP.

When viewed under fluorescence, the gGFP conidia captured onto Isopore filters provided sufficient contrast to be clearly seen and easily enumerated (Figure 1A). However, when viewed under white light these same conidia were visible only in a narrow depth of field and blended with the filter background making them much more difficult to identify and considerably more time consuming to count (Figure 1B). The comparison of identical fields of a randomly selected single filter using white light and fluorescent microscopy showed that some conidia were not visible using white light. Thus, fluorescent conidial counts were significantly higher compared to counts using white light ($p = 0.026$, based on a two-tail, paired t-test with a 95% confidence interval, n = 41 counting fields, mean of 4.1 conidia per counting field). Furthermore, counting times for a given filter were much longer (approximately 40%) using white light compared to fluorescent microscopy.

Replicate counts of the same filter were done on a subset of filters to evaluate variability associated with counting GFP-expressing conidia. The coefficient of variation for replicate fluorescent filter counts was 10% and agreed with the variability predicted by the Poisson distribution[27] (n = 4, mean of 2.3 conidia per counting field, mean of 77 fields counted). The respective halves on a subset of filters (n = 9) loaded with GFP expressing conidia in the aerosol chamber

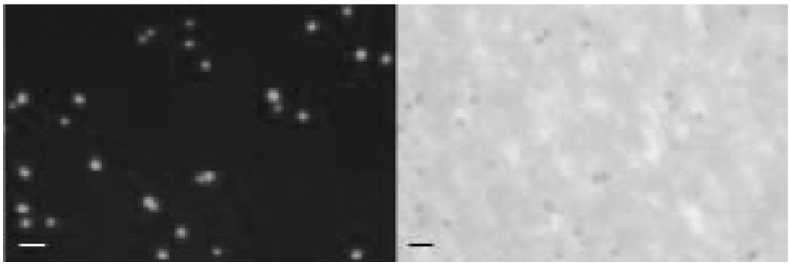

FIGURE 1. gGFP-expressing *A. fumigatus* conidia deposited on 0.8-μm Isopore polycarbonate filters viewed in the same field by epifluorescence using fluorescein filters (A) and under white light (B) at 400× magnification. Bars = 10 μm.

were compared using fluorescent counting for each filter half to ensure conidia were uniformly distributed. Box plots of the conidia count per field (square root transformed based on Poisson distribution) for each filter half showed similar distribution with respect to median, mean, and interquartile range (data not shown). The average number of conidia per field (square root transformed) counted on respective filter halves was not statistically different ($p = 0.3$) based on a two-tail, paired t-test with a 95% confidence interval ($n = 9$, mean of 7.8 conidia per counting field). Thus, one filter half can be counted optically and will provide a reliable estimate of the filter loading of the corresponding half that can be analyzed by alternative conventional or molecular means.

Test filters ($n = 38$) used to capture gGFP conidia in the aerosol test chamber and representing filter loadings ranging from 30–30,000 conidia per filter were split into equal halves and analyzed by either fluorescent microscopy or qPCR. A scatter plot of the data showed a linear relationship between qPCR and direct microscopic estimates of filter loading (Figure 2). A straight line was fit to the data using simple linear regression (Figure 2) with qPCR estimates of filter loading being the independent variable and direct microscopic counts of filter loading being the dependant variable ($y = 1.06\times + 404$; $R^2 = 0.91$). The 95% confidence interval for the slope ranged from 0.95 to 1.2, while the confidence interval for the intercept ranged from –215 to 1024. There were no patterns noted in the residual plot which would suggest violations of basic assumptions for normality (data not shown). There were a total of 7 reagent blanks and 10 filter blanks analyzed in addition to the 38 samples. No conidia were detected in any of the blank samples.

DISCUSSION

Techniques using qPCR have been proposed as an improvement over culture and microscopic methods traditionally used to enumerate fungal spores in air samples for the purpose of exposure assessment.[13] The use of filters for sample collection represents a traditional, well-validated sampling methodology familiar to the majority of industrial hygiene practitioners. The use of qPCR to analyze air samples collected on matrices, such as filters, is being marketed by commercial laboratories using EPA-licensed primers for environmental fungi.[22] However, qPCR sample processing and analysis protocols for airborne fungi have not been adequately validated in scientific peer reviewed literature. Haugland et al.[13] compared results of analyses of environmental samples of *S. chartarum* conidia using qPCR and optical (hemacytometer) methods using a liquid impinger collection process. However, the utility of this study is limited since few samples were collected, an internal standard control DNA was not used, and the reference standard was called into question.[13] Many of the published qPCR assays for *A. fumigatus* were developed for clinical applications, and validation of these assays typically involved recovering *A. fumigatus* DNA from clinical samples such as blood.[26,28–30]

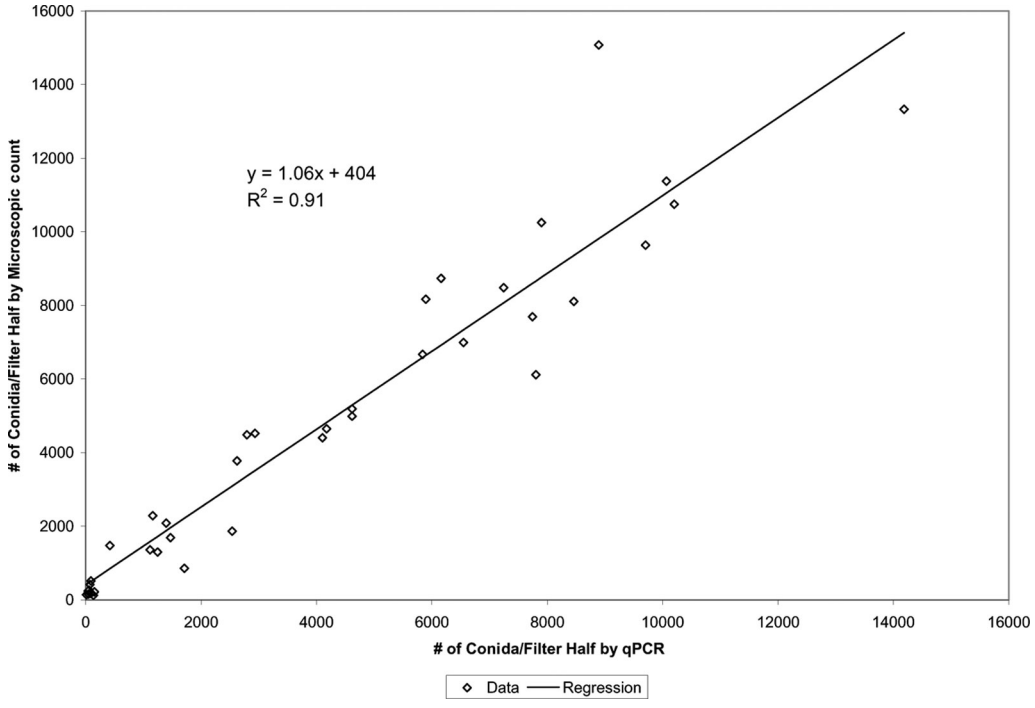

FIGURE 2. A scatter plot and regression line comparison of qPCR and direct microscopic estimates of filter loading ($n = 38$)

We have previously reported on a qPCR analysis method developed to detect *A. fumigatus* conidia optimized for environmental sampling that is highly specific for *A. fumigatus* DNA, can detect fewer than 10 conidia per PCR reaction, has linear response over a 4-$\log_{10}$ range, has low variability among replicate standards, incorporates an internal standard DNA control for detecting false negative results, and can be completed in less than 4 hours.[25] Validation of qPCR following collection of *A. fumigatus* conidia from air and removal from environmental sampling matrices such as filters has not been previously reported.

Comparison of newly developed methods with established methods is necessary to ensure that the new method is not biased and that results accurately reflect the actual numbers of conidia collected. Although there are no accepted benchmark methods for sampling airborne fungi to use as a comparison, culturable sampling methods such as the AGI-30 liquid impinger and Anderson samplers, which rely on culturing samples on growth media, have been suggested as reference methods.[15,31] However, the experiments performed as part of this investigation suggest that up to 40% of the conidia in stock cultures are not cultureable (data not shown). Therefore, methods relying on culture techniques would provide a biased underestimate for comparison, since nonculturable conidia would not be counted using culture-based sampling methods but would be included using molecular analysis.

While the enumeration of culturable conidia has implications for infectious disease, these issues relate only to a small portion of the general population, that is, persons who are immunocompromised. The methodology described here, however, has significantly broader applicability as the vast majority of health outcomes resulting from exposure to fungi, such as allergic hypersensitivity responses, are exacerbated by nonculturable as well as culturable conidia.

Testing in a laboratory-based aerosol test chamber allowed controlled filter loading by regulating *A. fumigatus* aerosol concentrations and sample collection time and flow rate. Thus, the collection and analysis method was evaluated over a range of conidial loads expected to be encountered during field sampling conditions. The use of this chamber also minimized external variables by ensuring only HEPA-filtered air and *A. fumigatus* conidia were introduced into the chamber. Consequently, other types of fungi or inhibitors were not being collected on the filter surface and, therefore, could not affect the results from qPCR analysis.

Microscopic counting of *A. fumigatus* GFP-expressing conidia on Isopore filters provided a direct means of quantifying filter loading. Direct counting of random, representative filter areas provided a method to accurately estimate the number of conidia present on the entire filter. The filter-counting procedures were based on methods developed and validated by the National Institute for Occupational Safety and Health for counting asbestos and synthetic vitreous fibers deposited on filters.[27] Although *A. fumigatus* conidia were visible using standard white light microscopy (Figure 1B), the use of white light resulted in significantly lower estimate of filter loading based on counts performed on a single, randomly selected filter. In addition, counting was time-consuming and technically challenging due to the lack of contrast between translucent conidia and the filter surface. Thus, direct counting under these conditions may induce error as well as decrease accuracy and precision. Conversely, GFP-expressing conidia were readily visible (Figure 1A), did not require special pretreatment (such as staining or tagging) or filter processing and, furthermore, were quantifiable using qPCR and remained stable for months.

The GFP protein has been used to illustrate protein structure and to mark gene expression and intracellular processes in many organisms.[23] Since some types of fungi do not efficiently express wild-type GFP,[32] Wasylnka and Moore[24] transformed *A. fumigatus* with a gGFP plasmid that had been optimized to provide a high level of expression in ascomycete fungi. In our study, this gGFP provided consistent and reliable fluorescence which facilitated conidial counting on Isopore filters. This type of GFP-expressing *A. fumigatus* does not occur naturally and was used as a tool for validation of sampling and analysis purposes only. Naturally occurring *A. fumigatus*, as well as most other environmental fungi, do not fluoresce and are not amenable to direct optical fluorescence counting when environmental samples are collected on filters.

The use of gGFP *A. fumigatus* enabled fluorescing conidia on Isopore filters to be reliably counted. This permitted the filters used to capture airborne conidia to be cut in half and the respective halves to be analyzed by either direct counting or qPCR. Variables such as conidia concentration, sampling location within the chamber, and temperature were negated during filtration since each filter was divided for analysis (i.e., each filter half was its own matched control). It can be stated with statistical confidence, based on the regression analysis of the test chamber samples (Figure 2), that there is a 1:1 relationship between the two enumeration methods (i.e., the confidence intervals around the slope of the line include 1). Thus, qPCR provides an accurate estimate of filter loading based on comparison with direct counting. Although the confidence interval around the intercept includes zero, one must consider the variability around the intercept when low numbers of conidia are collected on filters.

Based on physical characteristics, including smooth surfaces and straight-through pores of uniform size,[17] Isopore filters are compatible with the conidial recovery, digestion, and qPCR methods used as part of this investigation. When using 0.8 μm straight-through pore membrane filters, conidia, due to their size (approximately 2.5 μm), are captured primarily by impaction and interception with very high efficiency.[17] Since conidial capture occurs strictly on the filter surface, recovery efficiency from polycarbonate filters is high and greater than 90% recoveries have been reported.[33] Due to the hydrophobic nature of polycarbonate filters, water absorption and retention are low. Therefore, DNA, which is highly water soluble, will not be lost to the filter due to absorption of aqueous digestion buffers during DNA extraction from isolated conidia. The filters are also suited for molecular applications since they have

a low affinity for protein binding, are biologically inert, and have low extractables.[34]

This investigation validated the use of qPCR to estimate the number of airborne conidia deposited on filters in a clean laboratory environment. When environmental samples are processed, known and appropriate amounts of internal standard DNA must be incorporated into the qPCR analysis to detect inhibition of the PCR reaction. The addition of internal standard DNA to the PCR reaction prevents reporting of biased or false negative qPCR results and should be incorporated into all environmental qPCR analysis protocols.[25] The presence of inhibitors may prevent quantitative reporting of results unless samples are further processed for inhibitor removal and DNA purification.[12,25]

CONCLUSIONS

We show the use of GFP-expressing organisms effectively provides external validation of molecular enumeration methods. The use of GFP allows rapid and accurate fluorescent microscopic enumeration under conditions that may result in inaccurate conidial enumeration when using standard white light microscopy. We describe how the outlined qPCR analysis method is in agreement with an external reference method (microscopic counting) and is reliable for enumerating fungal conidia collected on filters. The use of qPCR provides sensitive, specific and accurate analysis of conidial DNA in less than 4 hours and can be used with traditional filter collection air sampling methods. Although the confirmation protocols used in this study were developed and used with a GFP-expressing variant of *A. fumigatus*, this model method is amenable to modification for use with other relevant environmental fungi and other microorganisms.

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