Detecting Low Concentrations of Airborne *Asp/Pen* Spores and *Aspergillus* Spp. in HEPA-Filtered Hospital Air Using Filtration, Microscopy and qPCR Analysis

September, 2009

Joe Spurgeon, Ph.D., CIH; Bayshore Environmental. Fullerton, CA [cih@bi-air.com]
Gregory Spahr, ME; Health and Safety Resources, Los Angeles, CA

ABSTRACT

A sampling protocol is described for detecting low concentrations of both airborne spores and fungi in HEPA-filtered hospital air. The 25 mm Bi-Air (BA) filter cassette was used to collect duplicate sample traces on 25 mm mixed cellulose ester (MCE) filters, concentrating the sample by a factor of 20.6 compared to standard 25 mm cassettes. One sample trace was analyzed for *Asp/Pen*-like spores by microscopy at 600X magnification. The second duplicate sample trace was aechived; and submitted for quantitative PCR (qPCR) analysis if *Asp/Pen*-like spores were detected. Microscopy provided a rapid method for pre-screening critical-care areas of healthcare facilities for *Asp/Pen*-like spores. The qPCR method identified *Asp/Pen*-like spores with sufficient sensitivity to detect *Aspergillus* spp. at the concentrations routinely collected in HEPA-filtered hospital air.

MCE filters were suitable for the detection of low concentrations of fungal spores when the samples were analyzed by both microscopy and qPCR. The results for microscopy were equivalent to qPCR in the range of 10-42 spores/sample and 10-263 spore equivalents (sp-eq) per sample, with a coefficient of correlation (r-value) of 0.99. Samples were collected in operating rooms, intensive care units, post-operative recovery, and surgical support areas of seven hospitals. The geometric mean (GM) concentrations for *Asp/Pen*-like spores ranged from 0.8 spores/m^3 to 2.8 spores/m^3 by microscopy and from 1 sp-eq/m^3 to 225 sp-eq/m^3 by qPCR.

The method was successfully used for baseline sampling, incident investigation, and post-remediation clearance sampling. Following development of the method, the field samples were routinely submitted to a commercial laboratory for microscopic analysis.

INTRODUCTION

Background

A sampling protocol for the detection of low concentrations of both airborne *Aspergillus /Penicillium* (*Asp/Pen*)-like fungal spores and *Aspergillus* species in HEPA-filtered hospital air is described. The sampling method involved the use of the Bi-Air (BA) filter cassette to collect duplicate sample traces on 25 mm mixed cellulose ester (MCE) filter media (Spurgeon 2006; Spurgeon 2007). One sample trace was analyzed by microscopy for the presence of *Asp/Pen*-like fungal spores. The second sample trace was submitted for analysis by quantitative PCR (qPCR) using a panel of 23 fungal primer pairs. The qPCR method was used to rapidly and accurately identify spores to species in those samples in which *Asp/Pen*-like spores were detected by microscopy.

The use of filter cassettes allowed airborne samples to be collected over extended periods of time, resulting in less variable TWA samples, potentially reducing false negatives, and lowering the limit of detection (LOD). The total area of a standard 25 mm filter cassette is 490 mm^2, with an exposed area of 385 mm^2 (7400 Method, NIOSH Methods of Analysis). The area of each BA sample trace was 9.35 mm^2, with a total exposed filter area of 18.7 mm^2. This area represented 4.9 % of the filter area exposed in a standard 25 mm cassette, thereby concentrating the sample by a factor of 20.6 compared to a standard 25 mm cassette (Spurgeon 2006).

It is well known that elevated concentrations of *Aspergillus* species (spp.) such as *A. fumigatus, A. flavus, A. terreus*, and *A. niger*, when present in critical-care areas of hospitals, may result in an increased risk of infection in immuno-compromised patients (Kordbacheh et al. 2005; Lee et al. 2007). The typical protocols for sampling *Aspergillus* spp. in healthcare facilities have emphasized the collection of culturable samples (Metha 1990; Morris et al. 2000; Falvey and Streifel 2007). However, culturable methods have several limitations for assessing patient exposures (Hay et al. 1995; Thio et al. 2000; Durand et al. 2002; McDevitt et al. 2004; Morrison et al. 2004; McDevitt et al. 2005). For example, the typically short sampling times may result in more variable results, they increase the likelihood of obtaining false negatives, and they result in a relatively high limit of detection (LOD). Culturable methods also have a delayed response time due to the need to culture the sample, which can increase patient risk during incident investigations. In addition, culturable methods only detect culturable structures rather than total spores. This bias can make the detection of fungal amplification sites and exposure pathways more difficult during incident investigations.

Several studies have concluded that longer sample times and/or larger air sample volumes were superior to small-volume samples for detecting *Aspergillus* spp. in the healthcare environment. Morris et al. (2000) concluded that longer sampling times were beneficial for estimating human exposure as part of an epidemiological investigation. Thio et al. (2000) compared the ability of large-volume (1,200 L) and small-volume (160 L) culturable air samples to detect *Aspergillus* spp. None of the 78 small-volume cultured samples grew an *Aspergillus* sp., while 10 of 40 large-volume cultured samples grew an *Aspergillus* sp. Similarly, Meklin et al. (2007), in a study using qPCR rather than culturable methods, also concluded that the interpretation of short-

term samples was less reliable compared to long-term sample results.

McDevitt et al. (2004) concluded that there was a need to develop sampling methods for pathogenic fungi that were more representative of patient exposures. They investigated the collection of time-weighted average (TWA) samples on 25 mm polycarbonate filters followed by qPCR analysis. McDevitt et al. (2005) reported that qPCR analysis was a sensitive and accurate method for detecting pathogenic fungi for sample loadings between 15 and 30,000 conidia per filter. They also reported a linear relationship between epifluorescent microscopy and qPCR in this concentration range. However, light microscopy was reported to be inaccurate , resulting in a lower estimate of filter loading compared to the qPCR method. In addition, microscopic analysis of 25 mm filters was reported to be time consuming, which would limit the methods adoption by commercial laboratories for routine analysis. McDevitt et al (2004) cut the 25 mm filter in half and then analyzed one of the filter halves with epiflourescent microscopy by analyzing a maximum of 100 microscopic fields of view (FOV), each with an area of 0.0625 mm^2. Therefore, only 1.6 % of the exposed filter area was analyzed. When attempting to detect 10-20 spores per sample, analyzing less than 2 % of the sample may represent a substantial limitation.

Purpose

One objective was to determine if concentrating the sample by a factor of 20 by using the BA filter cassette instead of a standard 25 mm cassette would reduce the sample-analysis time and improve the accuracy of the microscopic analysis.

The retention of fungal spores within a defined area on polycarbonate filter was difficult to achieve in a previous study, limiting the utility of this filter material for microscopic analysis using the BA (Spurgeon 2006). Therefore, a second objective was to determine if MCE filter media, which is suitable for the microscopy of fungal spores, was also suitable for qPCR analysis. The ability of MCE media to release spores quantitatively at low filter loadings, and the possible presence of microbial DNA in the cellulosic material were potential limitations.

A third objective was to determine if bright field microscopy could be used for the routine pre-screening of healthcare facilities for *Asp/Pen*-like spores. The qPCR method is a rapid, accurate and sensitive method for the identification and quantitative analysis of airborne spores, although it is still relatively expensive for routine use. Therefore, the correlation between microscopy and qPCR was used to assess the utility of microscopy for filter loadings both more and less than 10 *Asp/Pen*-like spores per sample.

The primary purpose of the study was to develop a method for routinely sampling and analyzing the air in HEPA-filtered hospital environments. However, a secondary objective was to demonstrate that commercial laboratories could routinely analyze BA samples by microscopy. This was demonstrated through the routine submission of samples to a commercial laboratory for microscopic analysis following the development phase of this study.
A fourth objective was to determine typical baseline concentrations of *Asp/Pen*-like spores and *Aspergillus* spp. in HEPA-filtered areas of hospitals using the BA protocol. These critical-care

areas included operating rooms (OR), organ transplant intensive care units (ICU), post-operative recovery (Post-Op), and surgical support (MRI, Oncology).

MATERIALS AND METHODS

Sample Collection

The BA cassettes (Bi-Air Corp, Placentia, CA) contained a 25 mm (MCE) filter (Millipore Corp, Bedford, MA) with a pore size of 3.0 um, which was supported by a cellulose pad. Samples were collected using a Model 224-PCXR8 (SKC, Inc., Eighty Four, PA), a Basic 12 (A. P. Buck, Inc., Orlando, FL), or a high-volume Gast pump (EMS, Inc., Charleston, SC). Depending on the objective, samples were collected at airflow rates between 1-3 lpm and for 2 - 4 hours, with a maximum sampling time of 7 hours. Total sample volumes varied from 120 liters to 1,260 liters. When both sample traces were analyzed by microscopy, the LOD varied from 8.3 spores/m^3 to 0.8 spores/m^3, respectively.

Microscopic Analysis

Following sample collection, the cassette was opened and the filter and support pad were removed with tweezers, with the filter placed sample-up on the pad. The two sample traces were separated by cutting the filter in half using an Xacto Knife with a ½ inch blade. Half the filter, containing one of the sample traces (50 % of the total sample collected) was placed on a glass slide sample-up and the excess filter material was trimmed away as illustrated in Photo 1, leaving the sample trace and a small area of surrounding filter media for microscopic analysis. The sample trace was then inserted into a Quick Fix® acetone vaporizer (EMS, Inc., Charleston, SC) to collapse and clear the MCE filter, stained with lactophenol cotton blue, covered with a cover slip, and analyzed by light microscopy. Spore concentrations were calculated by attributing 50 % of the total sample volume to each sample trace (Spurgeon 2006).

Analyses were performed using either a Nikon Labophot-2 or Alphaphot PCM microscope (Nikon America, NY, NY) equipped with 20x, 40x Phase Contrast, and 60x objectives. The size of a BA sample trace is 1.7 mm x 5.5 mm (Spurgeon 2006). Assuming that a microscopic field of view (FOV) at 600X magnification has a diameter of 0.28 mm, these dimensions are equivalent to 6 x 20 FOV. One entire sample trace, equivalent to about 120 FOV, was analyzed by microscopy at 600X magnification. Lactophenol cotton blue stain was used as the wetting agent to assist in differentiating between biological material and particulate matter. In addition, the Phase Contrast objective was sometimes used to further differentiate between biological material and particulate matter.

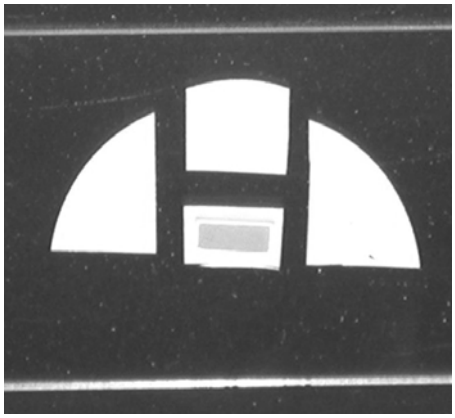

Photo 1. BA sample trace placed on a glass slide with the excess filter media trimmed away, showing the rectangular 4 % of the filter area retained for analysis.

qPCR Analysis

When a qPCR analysis was to be performed on a sample, one sample trace was placed sample-down on a clean glass slide, covered with a support pad, and the support pad taped to the slide using clear Scotch brand tape. The area of the sample trace was depressed below the surface of the filter, preventing the sample from coming in contact with the glass slide. The labeled slide was placed in a clean zip-lock plastic bag and shipped overnight to the laboratory for analysis.

The QPCR analyses were performed using the Applied Biosystems (ABI, Foster City, CA) Prism 7300 Sequence Detection System. Standard operating procedures were adapted from those developed by USEPA (National Exposure Research Laboratory, Cincinnatti, OH). All primer and probe sequences, as well as known species comprising the assay groups, have been published at the website: http://www.epa.gov/microbes/moldtech.htm; and reported by Haugland et al. (2004).

The archived MCE filter was removed from the glass slide using flame-sterilized tweezers and carefully inserted into a sterile 2 mL screw-capped DNA extraction tube pre-loaded with 0.7 mm Zirconia/Silica beads(S0201-50, GeneRite, NJ). Each sample was spiked with a 1 µL aliquot of *Geotrichum candidum* conidia suspension (1.75×10^4 spores) as a reference, and a 400 µL aliquot of lysis buffer (S2101-22, GeneRite) was added from a DNA-EZ extraction kit (K101-03C-50, GeneRite, NJ). The extraction tube was shaken in a bead beater (Biospec Products, Bartlesville, OK) for one minute at maximum speed, followed by one minute of centrifugation (16,110 x g) (Model 5415D, Eppendorf, Westbury, NY) at maximum rpm to pelletize the beads and debris. The crude extract was filtered through the pre-filter provided in the DNA-EZ kit to remove potential PCR interferences. The filtrate was mixed with 600 µL of binding buffer and applied to a DNAsure™ column (S5103-25C-50, GeneRite) followed by washing twice with EZ-washing buffer (S2301-50). The ultra pure DNA was obtained by eluting with elution buffer (S2401-50) provided in the DNA-EZ extraction kit. The detection sensitivity has been published elsewhere (Haugland 2004).

The equation supplied by Applied Biosystems (User Bulletin #2) was used to calculate the comparative cycle threshold (C_T) and for preparing standard calibration curves for target conidia or spore equivalents versus delta-cycle threshold values ($C_T = C_{T,target} - C_{T,reference}$) using co-extracted DNA from *G. candidum* as a reference (Haugland et. al. 2002, 2004). Although each spore of *Asp.* or *Pen*. only contains a single cell, the cells of some fungal species contain varying amounts of rDNA. Since pure conidia of each target species were used as a calibrator for the positive control and for both detection and quantification in each target assay, spore equivalents rather than cells was defined as the quantification unit for the target assay.

RESULTS

Table 1 contains the concentrations of sp-eq detected by qPCR for six MCE filters that were submitted as blanks. The data in Table 1 were obtained by analyzing the entire 25 mm filter. The columns in Table 1 are the sample number, total sp-eq per filter, sp-eq per 100 mm^2 of filter, the fungi detected in each sample, the concentration of each fungus, and the concentrations of these fungi contained in the 4 % of the filter area necessary for analysis by qPCR when analyzing a BA sample trace (Photo 1).

Table 1. Fungi detected on six 25 mm MCE filters submitted as qPCR blanks.

SAMPLE NUMBER	TOTAL SP EQ	SP EQ / 100 mm^2	FUNGI DETECTED	SP EQ / FILTER	SP EQ / TRACE
1	0	0	None Detected	0	0
2	0	0	None Detected	0	0
3	3	0.6	*Aspergillus Penicillioides*	3	0.1
4	27	5.5	*Penicillium brevicompactum*	20	0.8
			Asp. fumigatus	5	0.2
			Scopulariopsis Chartartum	2	0.1
5	37	7.6	*Cladosporium Herbarum*	18	0.7
			Mucor amphibiorum	12	0.5
			C. cladosporioides	7	0.3
6	156	31.8	*P. variabile*	63	2.4
			Aureobasidium pullulans	63	2.4
			Alternaria alternate	7	0.3
			Asp. Penicillioides	4	0.2

The 154 hospital samples described in Table 2 were analyzed by microscopy. Post-Op included post-operative recovery and surgical support areas such as MRI suite, catheter laboratory, Gamma Knife, radiation/oncology, and day surgery. Out Patient included patient waiting areas and emergency rooms, while Interior Spaces included lobbies, hallways, and offices. The parameters in Table 2 include sample size, number of samples below the LOD, the maximum *Asp/Pen* concentration detected, the geometric mean (GM) concentration, the geometric standard deviation (GSD), and the arithmetic average concentration. The sampled areas listed in the table are in descending order of GM concentrations, with GM concentrations in a relatively narrow range from a high of 2.8 spores/m^3 to a low of 0.8 spores/m^3. However, 60 % to 85 % of the samples collected in the various hospital areas and analyzed by microscopy had *Asp/Pen*-like

spore concentrations that were below the LOD for a particular sample.

Table 2. Concentrations of *Asp/Pen*-likespores (sp/m^3) for 154 samples collected in various areas of seven hospitals and analyzed by microscopy.

AREA	SAMPLES	BELOW LOD	MAX	GM	GSD	AVG
Post-Op	**10**	**6**	**78**	**2.8**	**9.6**	**17.4**
Interior Spaces	**43**	**30**	**222**	**2.1**	**9.4**	**24.7**
Out Patient	**19**	**13**	**108**	**1.6**	**2.7**	**13.6**
ICU	**62**	**47**	**218**	**1.1**	**4.5**	**6.9**
Operating Rooms	**20**	**17**	**15**	**0.8**	**2.7**	**1.7**

The data in Table 3 describe the sample results for 40 samples that were collected in seven hospitals and analyzed by qPCR using a panel of 23 primer pairs. Only the results for *Aspergillus* and *Penicillium* species are reported in the table. The sampled areas listed in the table are in descending order of GM concentrations. The GM concentration was highest in Post-Op, intermediate in Interior Spaces, and lowest in ICU's, OR's, and Out Patient. From 33 % to 50 % of the samples collected in the various hospital areas and analyzed by qPCR had *Aspergillus* and/or *Penicillium* concentrations that were below the LOD for a particular sample.

Table 3. Concentrations of *Aspergillus* and *Penicillium* spores (sp-eq/m^3) for 40 samples collected in various areas of seven hospitals and analyzed by qPCR.

AREA	SAMPLES	BELOW LOD	MAX	GM	GSD	AVG
Post-Op	**4**	**0**	**1,794**	**225**	**3.1**	**635**
Interior Spaces	**4**	**2**	**1,070**	**30.3**	**17.5**	**291**
ICU	**10**	**3**	**74**	**6.5**	**6.4**	**23.5**
Operating Rooms	**11**	**4**	**31**	**1.7**	**3.7**	**4.5**
Out Patient	**6**	**2**	**8**	**1.4**	**2.9**	**2.3**
Oncology	**5**	**3**	**9**	**1**	**3.5**	**2.3**

Figure 1 is a comparison of microscopy and qPCR for 32 samples with total *Aspergillus* and *Penicillium* concentrations less than 10 sp-eq per sample. For the 18 samples in which *Asp/Pen*-like spores were not detected by microscopy, sp-eq concentrations by qPCR were 0 in six samples, 1 or 2 in eight samples, and between 3 and 8 for four samples. The coefficient of correlation (r-value) for the 32 samples was 0.34.

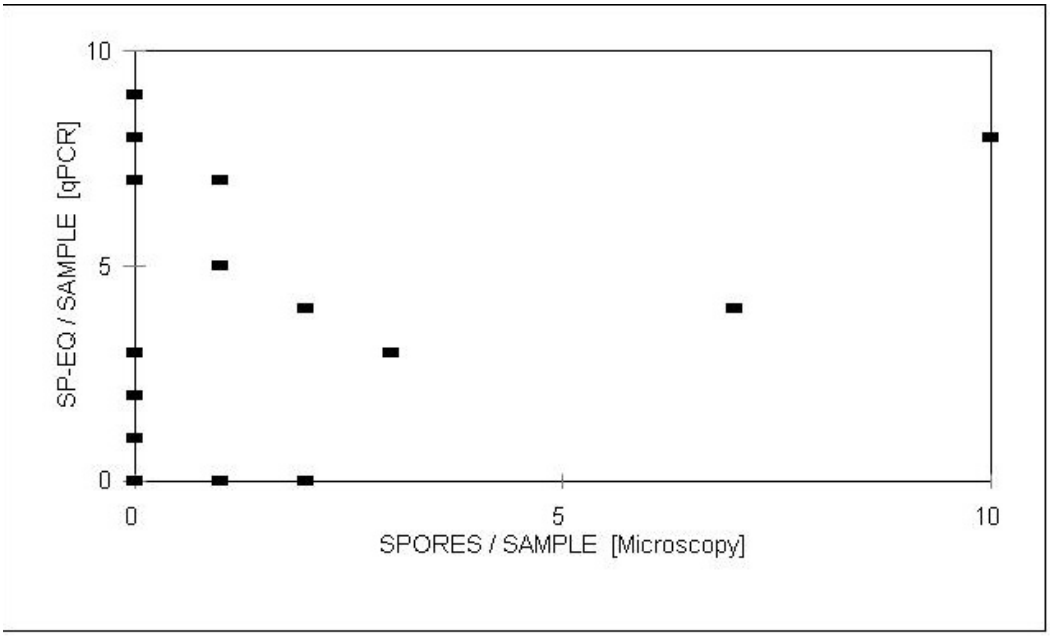

Figure 1. Comparison of microscopy and qPCR for 32 hospital samples with *Aspergillus-Penicillium* concentrations less than 10 sp-eq per sample. The data point at the origin represents 18 samples.

Figure 2 is a comparison of microscopy and qPCR for nine hospital samples with a total *Aspergillus* and *Penicillium* loading greater than 10 sp-eq per sample. Five samples had measurable *Aspergillus* and *Penicillium* concentrations although no spores were detected by microscopy. The coefficients of correlation (r-value) was 0.86.

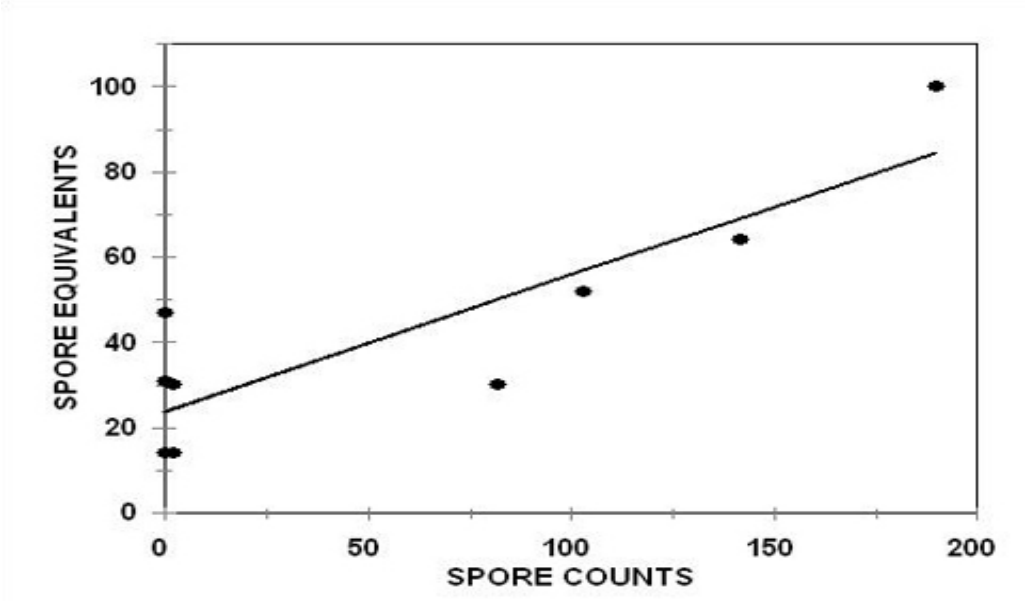

Figure 2. Comparison of qPCR and microscopy for nine hospital samples with *Aspergillus-Penicillium* concentrations greater than 10 sp-eq per sample.

DISCUSSION

Bi-Air Cassette

The collection of samples using the BA filter cassette offered several advantages compared to the collection of culturable samples on agar plates. First, the pre-screening of samples by microscopy provided a rapid method for detecting elevated concentrations of *Asp/Pen*-like spores, whereas culturable methods may not provide results for up to two weeks. Second, during incident investigations, sampling for total spores provided an advantage when evaluating pathways during an incident response. Not only were results available more rapidly, but sampling for total viable and nonviable spores rather than only viable spores was considered to be a more reliable approach for assessing exposure pathways and detecting the presence of fungal amplification sites. Third, since same-day results could be obtained, microscopy was the preferred method for analyzing post-remediation samples. Finally, pre-screening the samples by microscopy allowed the use of the more expensive qPCR method to be limited to (1) those archived samples in which elevated concentrations of *Asp/Pen*-like spores had been detected, and (2) when an assessment of patient risk was required. However, when identification to species was necessary, qPCR provided a specific, rapid, and sensitive method of analysis that could be applied to the archived BA filter samples.

Two characteristics of the BA were important in implementing this study. First, the BA produced two sample traces. The duplicate sample traces allowed the sample to be pre-screened by microscopy, and allowed quantitative methods of analysis to be applied to the duplicate samples. This limited the need for either the more expensive qPCR method and/or the slower culturable method. The use of the BA to collect duplicate TWA samples also resulted in a substantial reduction in project costs. For a specific LOD, a single TWA sample could replace the multiple short-term samples required to achieve that LOD.

Second, the design of the BA concentrated the sample 20-fold compared to a standard 25 mm cassette, making it possible to routinely analyze the entire BA sample trace. This allowed the detection of lower spore concentrations, reduced false negatives, and minimized both variability and analysis time. The 20-fold concentration of the sample by the BA allowed the results obtained by microscopy to be quantitatively compared to the qPCR results. For example, if a microscopic FOV were assumed to have an area of 0.063 mm^2, the filter area of 385 mm^2 exposed in a 25 mm cassette would be equivalent to 6,100 FOV; a slit impaction cassette with a 2 mm x 14.5 mm sample trace would be equivalent to 460 FOV; and a BA sample trace would be equivalent to 148 FOV. In addition, the smaller number of FOV in the BA sample made the method practical for routine analysis; and BA samples were routinely submitted to a commercial laboratory for microscopic analysis following the initial development of the method.

Potential Limitations

The presence of fungal DNA on blank MCE filters was a potential limitation in comparing airborne samples analyzed by both microscopy and qPCR. The concentrations of fungal sp-eq on six blank filters submitted for aPCR analysis, as described in Table 1, ranged from 0-156 sp-

eq per filter, with an average concentration of 37 sp-eq per 25 mm filter. These limited data suggested the expected average concentration of sp-eq per MCE filter would be 87 for a 37 mm filter; 19 for 50 % of a 25 mm filter; and 1.4 for a BA sample trace assuming 4 % of the filter was analyzed.

The small area of the BA sample trace provided an advantage by minimizing the amount of filter media required for qPCR analysis. Fungal DNA was not detected on two of the six blank filters (33 %). In addition, 16 of 42 field samples (38 %) did not contain detectable concentrations of sp-eq when the half-filters were analyzed by qPCR. Since special decontamination procedures were not used during the study, the source of the background DNA could not be determined. The amount of fungal DNA on the MCE media may have been variable, or it may have been randomly acquired during handling. First, although this was a limitation in the study design, these conditions were considered to be representative of actual field usage during routine sampling campaigns. Second, if *Asp/Pen* concentrations greater than 10 sp-eq per sample were interpreted as being significant to patient risk, as suggested by Figure 1, then the background sp-eq contained in the average BA sample trace would be 7-fold lower than this criterion.

A second potential limitation was the discrepancy between the results for microscopy and qPCR for some samples. *Asp-Pen* sp-eq were detected in 14 qPCR samples for which *Asp/Pen*-like spores were not detected by microscopy. Thirteen of these samples had a concentration of 1-7 sp-eq/sample, with one sample having a concentration of 31 sp-eq/sample. This result might be anticipated, since qPCR is a more sensitive method, with a greater ability to detect spore fragments that contain DNA. In addition, *Asp/Pen*-like spores were detected in four samples for which *Asp-Pen* sp-eq were not detected by qPCR. These samples had an *Asp/Pen* concentration of 1-2 spores/sample. One explanation for this discrepancy is that if total spore counts were one or two spores per sample, it was possible that all the captured spores would be collected in only one of the two sample traces. A second possibility is that the required primer pair was not included in the qPCR analysis. For example, either an atypical *Asp* or *Pen* was present, or the spore was not an *Asp/Pen*, but similar in appearance to *Asp/Pen*. Finally, the spore may not have been *Asp-Pen*, but reported as *Asp-Pen* like by microscopy.

A third potential limitation in comparing airborne samples analyzed by both microscopy and qPCR is that the results, when standardized for sample volume and reported per cubic meter of air, were not directly comparable for all fungi. Spore concentrations reported by microscopy were independent of spore type. For example, a single spore contained in a 168 liter sample, independent of spore type, was reported as a concentration of 6 spores/m^3. In the duplicate sample analyzed by qPCR, one sp-eq of *A. flavus* was reported as 6 sp-eq/m^3 while one sp-eq of *A. versicolor* was reported as 38 sp-eq/m^3. Different fungal species have different PCR amplification efficiencies that may be due to the following reasons: (1) Fungal spores of different species may have a different number of rDNA copies; (2) rDNA extraction efficiency varies from species to species due to the cell size and cell wall features; and (3) PCR inhibition or initial template copies may have also influenced PCR amplification efficiencies (ABI, Foster City, CA). These different PCR amplification efficiencies may impact the limits of detection. The values used in the EPA-licensed mold specific QPCR were obtained by performing QPCR reactions for each species with *Geotrichum candidum* as a standard, comparing the measured

number of cells with the known number of cells, and performing a statistical analysis. These factors may have contributed to a variable LOD for each fungal species identified by qPCR when the results were standardized by sample volume. Therefore, the direct comparison of microscopy and qPCR, as illustrated in Figures 1 and 2, was based on spore and sp-eq loadings per sample rather than concentrations per cubic meter.

McDevitt et al. (2005) reported a linear relationship between epifluorescent microscopy and qPCR for sample loadings between 15 and 30,000 conidia per filter. Figure 1 is a comparison of *Asp-Pen* spore concentrations reported by microscopy and qPCR for 32 hospital baseline samples with sp-eq concentrations of less than 10 sp-eq/sample. The substantial scatter and low coefficient of correlation (r-value) of 0.34 indicated a poor correlation between microscopy and qPCR for filter loadings in this range, which was consistent with the results reported by McDevitt. It was concluded that fungal loadings in this range were representative of typical conditions, and were classified as "background" concentrations. Little patient risk was expected to be associated with routine baseline samples in which the loadings were 10 sp-eq/sample or less. Therefore, once the study had progressed past the development phase and entered the implementation phase, these samples were typically not submitted for qPCR analysis.

A fourth potential limitation affecting qPCR analysis is the possible affect of the environment on the results. Although the r-values for both correlations in Figure 2 were 0.99, the regressions between the results for qPCR and microscopy were substantially different in hospital and residential environments. The correlation for the hospital samples had a slope of 5.55 and an intercept of 29.7, with qPCR substantially overestimating the spore loading compared to microscopy. However, since the hospital samples were collected in four different hospitals, these data suggest that hospital environments may be similar in their effect on qPCR results. The correlation for the residential samples, which were collected inside wall cavities, had a slope of 1.07 and an intercept of -9.8. A comparison of these two data sets indicated that microscopy and qPCR were highly correlated in both environments, although the response factor was substantially lower for the dusty residential samples. The hospital environments may have contained aerosolized drugs or other airborne promoters that affected the qPCR analysis. Since this factor was not identified until after the study was completed, the administration of aerosolized drugs during sample collection was not characterized, although it did occur. However, it was more likely that the sampled dust and debris may have contained qPCR inhibitors. Williams et al. (2001) reported a reduced qPCR sensitivity in the presence of pollen and dust in airborne samples. Dillon et al. (2007) also discussed the possible presence of qPCR inhibitors in airborne samples. Therefore, it may be prudent to characterize an environment (healthcare, residential, commercial, etc.) prior to applying correlations based on qPCR analysis.

A fifth potential limitation was that the qPCR analyses were performed using a limited set of 23 fungal primer pairs pre-selected for hospital environments. This limited the methods ability to detect a wide variety of fungi. However, since the emphasis in the hospital study was on the detection of a limited number of *Aspergillus* spp. and other infectious fungi, this was not considered to be a substantial limitation. However, one residential sample that was analyzed using the limited panel of 23 primer pairs had an *Asp/Pen*-like spore concentration of about 10,000 spores/m^3, although no *Asp-Pen* sp-eq were reported by qPCR. Therefore, in general, it

may be prudent to pre-screen samples by microscopy prior to submitting them for qPCR analysis.

Finally, the ability of MCE filter media to release spores quantitatively at low filter loadings was also a potential limitation to qPCR analysis. The data for the hospital samples in Figures 1 and 2 indicated the results for microscopy were correlated with qPCR in the range of 10 to 42 spores/sample and 10 to 263 sp-eq/ sample. Second, these data indicated that concentrating the spores by a factor of 20.6 with the BA cassette allowed low spore concentrations to be analyzed quantitatively using microscopy at 600X magnification. Therefore, the BA protocol had sufficient sensitivity to allow critical-care areas to be characterized as either typical or atypical by microscopy. Third, the correlations for both hospitals and residences indicated that qPCR analyses performed on samples collected on MCE filter media were in good agreement with microscopy. Therefore, apparently neither poor spore-release by the MCE nor the presence of fungal contaminants in the filter media adversely affected these comparisons.

A corollary to the ability of MCE media to release spores quantitatively for qPCR analysis is the ability of the MCE media to retain those same spores during shipment of the cassette. The good agreement obtained between microscopy and qPCR for the data sets illustrated in Figure 2 indicate that the loss of sample during shipment was inconsequential, since an r-value of 0.99 was obtained even after shipping the qPCR samples from coast-to-coast.

Sampling Protocol

One potential criticism of the sampling protocol was the reliance on total fungal spores rather than viable fungi to assess hospital environments. The protocol included an initial exposure assessment followed by a risk assessment. The presence or absence of detectable concentrations of *Asp-Pen* like spores represented the exposure assessment. If *Asp-Pen* like spores were not detected, then no exposure was occurring and a patient-risk assessment was not required. If *Asp-Pen* like spores were detected, then a risk assessment could be performed by either submitting the duplicate sample for culturing and/or analysis by qPCR.

The BA protocol included the quantitative pre-screening of samples by microscopy followed by the analysis of selected samples by qPCR. The sampling protocol was simple to implement in the field, provided rapid results, could rapidly identify fungi to species when necessary, and was cost-effective for routine sampling. The protocol was used for baseline sampling, incident investigation, and post-remediation clearance sampling.

The ability of the BA protocol to routinely collect TWA samples and to detect contaminant spores at a concentration of less than 10 spores/m^3 was considered to be a critical advantage, since the task of detecting contaminant spores in HEPA-filtered hospital air is often one of detecting a “rare event”. Microscopy provided a rapid method for pre-screening HEPA-filtered air for *Asp-Pen* like spores when the sample had been concentrated 20-fold by the BA. The upper graph in Figure 2 is the correlation between spores and sp-eq reported by microscopy and qPCR for four baseline hospital samples. Although only based on four samples, the r-value of 0.99 indicated that the results for microscopy at 600 X magnification were highly correlated with

qPCR in this range of sample loadings, which was consistent with the general results reported by McDevitt et al. (2005). The qPCR method provided a rapid, specific, and sensitive method for detecting medically important fungi. It was concluded that the BA sampling protocol may be suitable as a method for pre-screening critical-care areas of hospitals for airborne fungal contaminants.

Characterization of Hospital Environments

Baseline samples were typically collected at airflow rates of 1-2 lpm for periods of 2-3 hours. The minimum sample volume was 120 liters, resulting in a maximum LOD of 8.3 spores/m^3. Using the midpoints of these ranges, the typical LOD for baseline samples was about 4 spores/m^3. Sampling parameters for incident response investigations were typically an airflow rate of 3 lpm with sample times of 3 hours, resulting in a typical LOD of about 2 spores/m^3. Following a mold remediation, post-remediation samples were collected at an airflow rate of 3 lpm for a period of 7 hours, resulting in a LOD of 0.8 spores/m^3.

Many of the airborne samples collected in highly filtered air were below the LOD for a particular sample. Since excluding these data would have both biased the results and limited the sample size available for comparison, the data were treated as having been drawn from a lognormal distribution of concentrations. Data below the LOD were entered into the database as the LOD/2. Therefore, the reported geometric mean (GM) concentrations of *Asp/Pen*-like spores reported for some critical-care areas of hospitals, such as OR's, are artificially high because 65 % to 85 % of the data were below the LOD.

One objective was to determine typical baseline concentrations of *Asp/Pen*-like spores and *Aspergillus* spp. in HEPA-filtered areas of hospitals using the BA protocol. The data in Table 2 are spore counts (spores/m^3) measured by microscopy for representative areas sampled in seven hospitals. The GM concentrations of *Asp/Pen*-like spores were essentially 2 spores/m^3 or less in Sterile Processing, Operating Rooms, ICU's, Out Patient, and Interior Spaces; and less than 3 spores/m^3 in Post-Op and Surgical Support.

The data in Table 3 are a comparison of sp-eq/m^3 as measured by qPCR for similar areas in these seven hospitals. The GM concentrations of *Aspergillus* and *Penicillum* (sp-eq/m^3) in Sterile Processing, Oncology, Out Patient areas, and Operating Rooms were less than 2 sp-eq/m^3; the GM concentration in ICU's was 6.5 sp-eq/m^3; and Interior Spaces had a GM of 30.3 sp-eq/m^3. However, Post-Op and Surgical Support had a relatively high GM of 225 sp-eq/m^3. This may have been due to the number of visitors and visitor activity typically present in Post-Op environments.

Curtis et al (2005) reported a mean *Aspergillus* concentration of 30.6 cfu/m^3 for organ transplant ICU's (3). The organ transplant ICUs included in this study had a mean *Aspergillus* concentration of 23.5 sp-eq/m^3. However, Curtis et al reported that 40 % of their samples were positive for *Aspergillus*, while 70 % of the samples in the current study were positive for *Aspergillus*. Therefore, qPCR may be somewhat more sensitive compared to culturable methods

for detecting relatively low concentrations of *Aspergillus*.

Representative Field Investigations

An example of an incident investigation is illustrated by a suspect Operating Room. Because physicians were reluctant to use the facility, a request was made by Infection Control to assess the OR for fungal contaminants. A 3-hour BA sample was collected at an airflow rate of 3 lpm, resulting in a LOD of 1.9 spores/m^3. Four *Asp/Pen*-like spores and one *Stachybotrys* spore were detected in the sample, confirming that a fungal amplification site was affecting the OR. First, this example illustrates the concept that the investigator is attempting to detect a "rare event" when sampling for contaminant spores in highly filtered air. This result was equivalent to detecting one contaminant spore an average of every 36 minutes; or one spore per 108 liters of sampled air. Second, the LOD required to detect the problem in this OR was quite low. *Stachybotrys* was detected at a concentration of 1.9 spores/m^3, and *Asp/Pen*-like spores were detected at a concentration of 7.6 spores/m^3. Unless an extended-period TWA sample had been collected, or multiple grab samples had been collected over an extended period of time, the probability of obtaining a false negative (failing to detect an existing problem) would have been greatly increased. As the result of the recommended inspection by Facilities following the investigation, two walls of the OR were found to be contaminated and were remediated. Due to the potential for false negatives and higher LOD, it is unlikely that these fungal amplification sites would have been detected if a typical short-term air sample had been collected.

An example of applying the described sampling protocol to post-remediation clearance sampling is illustrated by a mold remediation in an organ transplant ICU ward. The parties involved in the remediation agreed that a concentration of 0.8 spores/m^3 or less of *Asp/Pen*-like spores would be the acceptance criterion for airborne samples. This LOD required the collection of 1,260 liters of air per sample, which was collected at an airflow rate of 3 lpm over a period of seven hours using BA cassettes. A total of 13 sampling locations were required to assess the ICU ward. One room had an *Asp/Pen*-like spore concentration of 0.8 spores/m^3 and was failed. It was re-cleaned and then passed; requiring a total of 14 BA samples to be collected. In comparison, either nine 5-minute N6 culturable samples or nine 10-minute slit impaction samples would have been required at each of the 14 sampling locations in order to achieve an LOD of 0.8 spores/m^3. Collecting 14 BA samples versus 126 samples using an alternative sampling device provided a substantial cost advantage.

Curtis et al (2005) reported that *A. fumigatus* concentrations measured in rooms during duct cleaning suggested that air supply ducts may be a significant source of *A. fumigatus* spores, and fungal spores in general. Baseline airborne samples were collected in one hospital during Santa Ana winds (strong desert winds). Elevated *Asp/Pen*-like spores (280 spores/m^3) were detected in Post-Op by microscopy; and elevated *Asp* and *Pen* (1,794 sp-eq/m^3) by qPCR. The maximum *Asp/Pen*-like spore concentration measured by microscopy was 28 spores/m^3 in a second set of confirming samples collected several days later during normal weather conditions. Surface dust samples were then collected from the internal fiberglass insulation in both the hot and cold decks of the air delivery system and the variable air volume mixing boxes. The surface samples were analyzed by qPCR and the results reported as sp-eq/100 cm^2 of surface area. No *A. fumigatus*

was detected in the sample from the cold deck; 300 sp-eq/100 cm^2 of *A. fumigatus*, a thermo-tolerant fungus, was detected in the sample from the hot deck; and an average of 1,025 sp-eq/100 cm^2 was detected in four samples from mixing boxes. This anecdotal example suggests that the components of the air delivery system, as suggested by Curtis et al (2005), may be one source of airborne contaminant fungal spores; and that their contribution may be episodic and therefore difficult to detect.

SUMMARY

The Bi-Air filter cassette was used for the detection of airborne fungal spores and fungi in HEPA-filtered hospital air. The BA protocol included the quantitative pre-screening of samples by microscopy followed by the analysis of selected samples by qPCR. The sampling protocol was simple to implement in the field, provided rapid results, could rapidly identify fungi to species when necessary, and was cost-effective for routine sampling. The protocol was used for baseline sampling, incident investigation, and post-remediation clearance sampling.

MCE filters were suitable for the detection of low concentrations of fungal spores when the samples were analyzed by either microscopy or qPCR. The filter media had sufficient affinity for spores to physically retain them for microscopy, yet efficiently released them for quantitative analysis by qPCR. A linear relationship between qPCR and direct microscopic analysis was confirmed for filter loadings between 10-42 spores/sample and 10-263 spore equivalents (sp-eq) per sample, with a coefficient of correlation (r-value) of 0.99. However, there was little correlation ($r = 0.34$) between the two methods for background spore concentrations, which were typically in the range of 0-10 spores and/or sp-eq per sample.

Finally, concentrations of *Asp/Pen*-like spores and *Asp-Pen* species detected in OR, ICU, and Post-Op areas of seven hospitals are reported. The geometric mean (GM) concentrations for microscopy ranged from a 0.8 spores/m^3 to 2.8 spores/m^3. The GM concentrations by qPCR ranged from 1 sp-eq/m^3 to 225 sp-eq/m^3

REFERENCES

Curtis L, Call S, Conroy L, Baker K, Ou C-H, Hershow R, Norlock-Cruz F, Scheff P. (2005). Aspergillus surveillance project at a large tertiary-care hospital. J Hosp Infect. 59(1):188-196.

Dillon, H. K., D. K. Boling, and J. D. Miller (2007). Comparison of Detection Methods for *Aspergillus fumigatus* in Environmental Air Samples in an Occupational Environment. JOEH, 4:509-513.

Durand, K. T. H., M. L. Muilenberg, H. A. Burge, and N. S. Seixas (2002). Effect of Sampling Time on the Culturability of Airborne Fungi and Bacteria Sampled by Filtration. Ann. Occup. Hyg. 46(1):113-118.

Falvey, D. G., and A. J. Streifel (2007). Ten-year air sample analysis of *Aspergillus* prevalence in a university hospital. J. Hosp. Infect. 67(1):35-41.

Haugland, R. A., N. E. Brinkman, and S. J. Vesper (2002). Evaluation of rapid DNA extraction methods for the quantitative detection of fungal cells using real time PCR analysis. J. Microbiol. Meth. 50:319-23.

Haugland, R. A., M. Varma, L. J. Wymer, and S. J. Vesper (2004). Quantitative PCR of selected *Aspergillus, Penicillium* and *Paecilomyces* species. Sys. Appl. Microbiol. 27:198-210.

Hay, R. J., Y. M. Clayton, and J. M. Goodley (1995). Fungal Aerobiology: how, when and where? J. Hosp. Infect. 30(1):352-357.

Kordbacheh, P., F. Zani, P. Kamali, K. Ansari, M. Safara (2005). Study on the Sources of Nosocomial Fungal Infections at Intensive Care Unit and Transplant Wards at a Teaching Hospital in Tehran. Iranian J. Publ. Health. 34(2):1-8.

Lee, L. D., M. Berkheiser, Y. Jiang, B. Hackett, R. Y. Hachem, R. F. Chemaly and I. I. Raad (2007). Risk of Bioaerosol Contamination With *Aspergillus* Species Before and After Cleaning Rooms Filtered With High-Efficiency Particulate Air Filters That House Patients With Hematologic Malignancy. Infect. Control and Hosp. Epidemiology. 28(9):1066-1070.

McDevitt, J. J., P. S. J. Lees, W. G. Merz, and K. L. Schwab (2004). Development of a method to detect and quantify *Aspergillus fumigatus* conidia by quantitative PCR for environmental air samples. Mycopathologia. 158:325-335.

McDevitt, J. J., P. S. J. Lees, W. G. Merz, and K. J. Schwab (2005). Use of Green Fluorescent Protein-Expressing Aspergillus fumigatus conidia to Validate Quantitative PCR Analysis of Air Samples Collected on Filters. JOEH. 2(12):633-640.

McGrath, J. J., W. C. Wong, J. D. Cooley, and D. C. Straus (1999). Continually measured fungal profiles in sick building syndrome. Curr. Microbiol. 38(1):33-6.

Meklin, T., T. Reponen, C. McKinstry, S-H Cho, S. A. Grinsphun, A. Nevalainen, A. Vepsalainen, R. A. Haugland, G. LeMasters, and S. J. Vesper (2007). Comparison of mold concentrations quantified by MSQPCR in indoor and outdoor air samples simultaneously. Science of the Total Environment. 382:130-134.

Metha, G. (1990). Aspergillus endocarditis after open heart surgery: an epidemiological investigation. J. Hosp. Infect. 15(3):245-253.

Morris, G., M. H. Kokki, K. Amderson, and M. D. Richardson (2000). Sampling of Aspergillus spores in air. J. Hosp. Infect. 44(1): 81-92.

Morrison, J., C. Yang, K.-T. Lin, R. A. Haugland, A. N. Neely and S. J. Vesper (2004). Monitoring *Aspergillus* species by quantitative PCR during construction of multi-storey hospital building. . J. Hosp. Infect. 57:85-87.

Spurgeon, J. (2006). A New Filter Cassette for the Direct Microscopic Examination of Airborne Fungal Spores. Aerosol. Sci. & Tech. 40(11):1025-1033.

Spurgeon, J. (2007). A Comparison of Replicate Field Samples Collected with the Bi-Air, Air-O-Cell, and Graesby-Andersen N6 Bioaerosol Samplers. Aerosol Sci. & Tech. 41(7):761-769.

Thio, C. L., D. Smith, W. G. Merz, A. J. Streifel, G. Bova, L. Gay, C. B.Miller, and T. M. Perl (2000). Refinements of environmental assessment during an outbreak investigation of invasive aspergillosis in a leukemia and bone marrow transplant unit. Infect. Control Hosp. Epidemiol. 21(1):18-23.

Williams, R. H., E. Ward and H. A. McCartney (2001). Methods for Integrated Air Sampling and DNA Analysis for Detection of Airborne Fungal Spores. Appl. and Environ. Microbiology. June: 2453-2459.