PAPER

Quantitative PCR analysis of house dust can reveal abnormal mold conditions†

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Indoor mold concentrations were measured in the dust of moldy homes (MH) and reference homes (RH) by quantitative PCR (QPCR) assays for 82 species or related groups of species (assay groups). About 70% of the species and groups were never or only rarely detected. The ratios (MH geometric mean : RH geometric mean) for 6 commonly detected species (*Aspergillus ochraceus*, *A. penicillioides*, *A. unguis*, *A. versicolor*, *Eurotium* group, and *Cladosporium sphaerospermum*) were >1 (Group I). Logistic regression analysis of the sum of the logs of the concentrations of Group I species resulted in a 95% probability for separating MH from RH. These results suggest that it may be possible to evaluate whether a home has an abnormal mold condition by quantifying a limited number of mold species in a dust sample. Also, four common species of *Aspergillus* were quantified by standard culturing procedures and their concentrations compared to QPCR results. Culturing underestimated the concentrations of these four species by 2 to 3 orders of magnitude compared to QPCR.

Introduction

Mold growth in homes, schools and other buildings has become a major issue of public concern. The use of a combination of methods, including visual inspections, moisture testing and culture- and/or microscopy-based microbial analyses of air and surface samples, is considered to be the best means of identifying building mold problems.[1,2] Performing all of these procedures can require considerable time, labor and expense, however, and their results are only as valid as the thoroughness and competence of the inspectors and analysts performing them. The development of more rapid, inexpensive and standardized methods for mold analysis and interpretation of the results could greatly contribute to reducing these costs and uncertainties.

Analyses of house dust samples have been suggested to provide a better indication of cumulative exposures to molds than short-duration air samples.[1,3] Findings of gradual increases in mold concentrations in floor dust, despite regular vacuuming,[4] suggest that this matrix may serve as a reservoir of fungal contamination. A DNA-based method for quantitative measurement of different species or closely related groups of indoor molds has been developed at the United States Environmental Protection Agency[5] and has been used for the analysis of selected target organisms in environmental samples, including building dust.[6–10] In this study, 82 QPCR assays were applied to quantify indoor molds in dust samples from MH and RH and the data were used to develop a prototype logistic regression analysis-based approach for the presumptive differentiation of these two categories of homes. Four species of *Aspergillus* were also quantified by widely used culture-based analysis and the results compared to QPCR analysis.

Experimental

Selection of homes and dust sampling

The study homes were selected in a larger, ongoing study in Cincinnati, OH on the interactions between diesel exhausts, aeroallergens, genetics and atopy on children's health. Information on housing characteristics and conditions was collected during walk-through investigations of the study homes and from questionnaires provided to the occupants. In addition, any visible moisture and mold damage in the homes was recorded.

Eighteen homes, having a total area of at least 0.2 m^2 of visible mold growth, were selected as moldy-homes (MH) and 19 reference homes (RH) homes were selected randomly from those with no visible moisture or mold damage or mold damage history. During a walk-through, dust samples were collected from the floor of the primary activity room of the child participating in the larger study. Samples were collected by vacuuming with a Filter Queen Majestic® vacuum cleaner (Health-Mor, HMI Industries Inc., Seven Hills, OH) for 2 min m^{-2} of floor area sampled. Total sampling area was 2 m^2 for carpets. For hard floors, the entire open floor area in the room was sampled. Information on the area and the material vacuumed was recorded. Dust samples were sifted (355 μm sieve) and the fine dust was stored at -20 °C.

Fungal cultures and rDNA sequences

Species names, culture collection sources and relevant GenBank sequence accession numbers of standard cultures used for the QPCR assays are listed in Table 1. The species

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Table 1 Fungal cultures, rDNA sequences, QPCR assays and calibration standard curve parameters used for target organism quantification

Standard cultures and rDNA sequences	QPCR assays and standard curve parameter values		
Species, strain, GenBank accession #	Assay name	Slope (*b*)	*y*-intercept (*a*)
Acremonium strictum, ATCC 34717, *cf*. AY625058	Astrc	−3.73	23.83
Alternaria alternata, EGS 35-193, *cf*. AY625056	Aaltr	−4.00	23.76
Aspergillus caespitosus, SRRC 308, AY373841	Acaes	−3.53	19.85
Aspergillus candidus, NRRL 303, *cf.* AY373842	Acand3	−3.39	20.23
Aspergillus carbonarius, SRRC 15, AY373844	Acarb	−3.58	20.40
Aspergillus cervinus, SRRC 371, AY373845	Acerv	−4.23	32.73
Aspergillus clavatus, SRRC 17, *cf.* AY373845	Aclav	−3.92	26.33
Aspergillus flavipes, NRRL 302, *cf.* AY373849	Aflvp2	−3.85	26.28
Aspergillus flavus, ATCC 16883, *cf.* AY373848	Aflav	−3.61	22.74
Aspergillus fumigatus, NRRL 163, *cf.* AY373851	Afumi	−3.56	20.96
Aspergillus niger, ATCC 16888, AY373852	Anigr	−3.38	21.01
Aspergillus niveus, SRRC 333, AY373853	Anive	−3.90	28.37
Aspergillus ochraceus, NRRL 398, AY373856	Aochr1	−3.60	23.44
Aspergillus paradoxus, SRRC 336, AY373860	Apard	−3.47	19.74
Aspergillus parasiticus, NRRL 502, AY373859	Apara	−3.50	24.07
Aspergillus penicillioides, ATCC 16910, AY373862	Apeni2	−3.55	23.13
Aspergillus puniceus, SRRC 2155, AY373862	Apuni	−3.53	22.04
Aspergillus restrictus, ATCC 16912, AY373864	Arest	−3.83	25.35
Aspergillus sclerotiorum, ATCC 16892, AY373866	Asclr	−3.56	22.30
Aspergillus sydowii, NRRL 250, AY373866	Asydo3	−3.39	25.29
Aspergillus tamarii, NRRL 427, *cf.* AY373870	Atama2	−3.67	24.12
Aspergillus terreus, ATCC 1012, AY373871	Aterr2	−3.46	23.06
Aspergillus unguis, SRRC 344, AY373872	Aungu	−3.29	21.16
Aspergillus ustus, NRRL 275, AY373877	Austs2	−3.45	21.09
Aspergillus versicolor, NRRL 238, *cf.* AY373882	Avers2	−3.71	27.24
Aspergillus wentii, NRRL 377, *cf.* AY373884	Awent	−4.63	30.76
Aureobasidium pullulans, EPA 701, AY625057	Apull	−3.38	20.93
Chaetomium globosum, ATCC 32404, *cf*. AY625061	Cglob	−3.52	20.28
Cladosporium cladosporioides 1, ATCC 6721, AY625059	Cclad1	−3.42	20.30
Cladosporium cladosporioides 2, ATCC 16022, AY625060	Cclad2	−3.42	19.75
Cladosporium herbarum, ATCC 28987, AY625062	Cherb	−3.53	19.00
Cladosporium sphaerospermum, UAMH 7686, AY625063	Cspha	−3.57	20.30
Emericella nidulans, NRRL 2395, AY373888	Anidu2	−3.69	24.32
Emericella variecolor, SRRC 268, AY373893	Avari	−3.92	24.65
Eurotium amstelodami, NRRL 90, AY373885	Eamst	−3.46	20.56
Epicoccum nigrum, UAMH 3247, AY625063	Enigr	−3.49	21.99
Memnoniella echinata, UAMH 6594, AF081470	Mem	−3.69	20.77
Mucor racemosus, NRRL 1428, AY625074	Muc1	−3.32	18.82
Paecilomyces variotii, ATCC 22319, AY373941	Pvari2	−3.45	20.97
Penicillium atramentosum, NRRL 795, AF033492	Patra	−3.77	25.82
Penicillium aurantiogriseum, FRR 971, AY380455	PenGrp1	−4.04	25.33
Penicillium brevicompactum, FRR 862, AY373898	Pbrev	−3.97	25.91
Penicillium canescens, FRR 910, AY373901	Pcane2	−3.81	27.02
Penicillium chrysogenum, EPA 467, AY373903	Pchry	−3.50	22.43
Penicillium citreonigrum, FRR 2046, AY373908	Pcteo	−3.51	29.15
Penicillium citrinum, FRR 1841, AY373904	Pcitr	−4.22	27.49
Penicillium coprophilum, NRRL 13627, AF033469	Pcopr	−3.85	29.03
Penicillium corylophilum, FRR 802, AY373906	Pcory	−3.72	24.36
Penicillium crustosum, FRR 1669, AY373907	PenGrp2	−4.27	27.46
Penicillium decumbens, FRR 741, AY373909	Pdecu2	−4.28	28.26
Penicillium digitatum, FRR 1313, AY373910	Pdigi	−3.63	22.82
Penicillium expansum, ATCC 7861, AY373912	Pexpa	−3.47	25.32
Penicillium fellutanum, FRR 746, AY373913	Pfell	−3.99	29.49
Penicillium glandicola, FRR 2036, AY373916	Pglan	−3.59	24.98
Penicillium griseofulvum, FRR 3571, AY373917	Pgris	−3.45	23.61
Penicillium implicatum, FRR 2061, AY380455	Pimpl	−3.62	22.17
Penicillium islandicum, NRRL 10127, *cf.* AY373919	Pisla	−3.49	22.22
Penicillium italicum, ATCC 48114, AY373920	Pital	−3.42	26.66
Penicillium melinii, FRR 2041, AY373923	Pmeli	−3.48	19.10
Penicillium miczynskii, FRR 1077, AY373924	Pmicz	−3.59	24.23
Penicillium olsonii, NRRL 28496, *cf.* AY373925	Polsn	−3.78	24.63
Penicillium oxalicum, NRRL 787, AF033438	Poxal	−3.96	25.74
Penicillium purpurogenum, FRR 1061, AY373926	Ppurp	−3.68	22.85
Penicillium raistrickii, FRR 1044, AY373927	Prais3	−3.58	23.42
Penicillium restrictum, NRRL 1748, AF033457	Prest2	−3.56	22.93
Penicillium roquefortii, FRR 849, AY373929	Proqu	−4.24	26.34
Penicillium sclerotiorum, FRR 2074, AY373930	Psclr	−3.32	19.48
Penicillium simplicissimum, NRRL 1075, AF033440	Psimp2	−3.42	23.74
Penicillium spinulosum, FRR 1750, AY373933	Pspin2	−3.32	24.81
Penicillium variabile, FRR 1290, AY373936	Pvarb2	−3.34	20.52
Rhizopus stolonifer, ATCC 14037, AY625075	Rstol	−3.62	18.51
Scopulariopsis brevicaulis, UAMH 7771, AY625065	SCbrv	−3.39	19.24
Scopulariopsis chartarum, ATCC 16279, AY625066	SCchr	−3.39	20.02
Stachybotrys chartarum, UAMH 6417, AF206273	Stac	−3.55	18.96

Table 1 Fungal cultures, rDNA sequences, QPCR assays and calibration standard curve parameters used for target organism quantification (*Continued*)

Standard cultures and rDNA sequences	QPCR assays and standard curve parameter values		
Species, strain, GenBank accession #	Assay name	Slope (*b*)	*y*-intercept (*a*)
Trichoderma asperellum, ATCC 38501, *cf.* AJ230669	Taspr1	−3.32	21.51
Trichoderma atroviride, EPA 405, AY625067	Tviri	−3.75	22.92
Trichoderma harzianum, NRRL 13019, AY625068	Tharz	−3.89	25.70
Trichoderma longibrachiatum, UAMH 9515, AY625069	Tlong	−3.42	22.68
Ulocladium atrum, EGS 30-188, *cf.* AY625072	Uatrm	−3.61	17.01
Ulocladium chartarum, EGS 36-055, *cf.* AY625071	Uchar	−3.63	22.46
Ulocladium botrytis, EGS 13-030, *cf.* AY625070	Ubotr	−3.58	18.75
Wallemia sebi, UAMH 7897, AY625073	Wsebi	−3.43	21.34

assignments were verified, or in some cases revised, from macro- and microscopic examinations of the cultures and by comparisons of their rDNA sequences with those of previously published sequences in GenBank.

QPCR assays and standard curves

Methods have been reported for preparing conidia or spore suspensions from fungal cultures, extracting DNA, performing QPCR analyses and preparing standard calibration curves for target conidia or spore equivalents *versus* delta cycle threshold values ($\Delta C_T = C_{T,target} - C_{T,reference}$), using co-extracted DNA from *Geotrichum candidum* as an exogenous reference.[6,9] Methods for estimating the amplification factors and extrapolating spore or conidia sensitivities of the assays from the standard curves have also been described.[6,9] All primer and probe sequences used in the assays as well as known species comprising the assay groups are at the website: www.epa.gov/nerlcwww/moldtech.htm. Primers and probes were synthesized commercially (Applied Biosystems, Foster City, CA; Integrated DNA Technologies, Coralville, IA; Sigma Genosys, Woodlands, TX).

DNA extractions and QPCR enumeration of molds

Mixed, positive control suspensions, containing approximately 10^4 or 10^5 spores or conidia ml^{-1} of each of the standard cultures listed in Table 1 were prepared as previously described.[9] Dust samples and positive control suspensions were extracted by a rapid bead-milling method.[8] Briefly, 90 μl of the suspensions or 5 mg of dust and 90 μl of AE buffer (Qiagen, Valencia, CA) were added together with 10 μl of a 2×10^8 conidia ml^{-1} reference suspension of *G. candidum* to sterile 2 ml conical bottom, screw cap tubes (506–636; PGC Scientifics, Gaithersburg, MD), containing 0.3 g of glass beads (G-1277; Sigma, St. Louis, MO) and 100 and 300 μl of lysis and binding buffer, respectively from an Elu-Quik DNA Purification Kit (Schleicher and Schuell, Keene, NH). The tubes were shaken in a Mini Bead-Beater (Biospec Products, Bartlesville, OH) for 1 min at a maximum speed and then centrifuged for 1 min at 8 000*g* to pellet the glass beads and debris. The supernatants were further purified using a DNeasy kit (Qiagen, Valencia, CA).

QPCR assays for target organism and *G. candidum* reference DNA in the extracts were prepared using a "Universal Master Mix" of PCR reagents (Applied Biosystems, Foster City, CA) and performed in an Applied Biosystems Prism model 7700 sequence detection instrument, as previously described.[6] Numbers of spores or conidia detected in dust samples (N) were calculated using the equation: $\log_{10}(N) = (\Delta C_T - a)/b$, where ΔC_T was the difference in observed C_T values between the target and reference organisms ($C_{T,target} - C_{T,reference}$) for the respective dust sample and a and b (Table 1) were the mean *y*-intercept and slope parameter values from the standard calibration curves for each target assay group. Parallel analyses of method negative control samples, containing AE buffer only, were performed at a frequency of approximately one per each six test samples analyzed. Mixed, positive control conidia suspensions were analyzed at a frequency of nearly one per each test sample.

Culture based analyses of molds

Fungal colony-forming units (CFU) were isolated from dust samples by standard culturing protocols.[11] Dust samples of 50 mg each were added to 15 ml sterile tubes (Corning Inc., Corning, NY) along with 4.5 ml of buffer (0.0425 g l^{-1} KH_2PO_4, 0.25 g l^{-1} $MgSO_4 \cdot 7H_2O$, 0.008 g l^{-1} NaOH 0.02% (v/v) Tween 80). The samples were shaken at 450 rpm (gyrotory® shaker, model G76, New Brunswick Scientific, Edison, NJ) for 1 h at room temperature. A series of dilutions were prepared for each sample and plated on 2% malt extract agar (MEA) (Difco, Becton Dickinson and Company, Sparks, MD) and dichloran-18-glycerol agar (DG18) (Oxoid LTD., Basingstoke, Hampshire, England) with chloramphenicol at a concentration of 100 mg l^{-1}. The samples were incubated at 25 °C for 7 days. Blank media and blank buffer cultivations were used for quality assurance. Colonies of *Aspergillus fumigatus*, *A. niger*, *A. ochraceus* and *A. versicolor* were identified based on colony appearance and species identifications were confirmed based on conidial structures and appearance using high resolution light microscopy (Labophot 2, Nikon Corp., Tokyo, Japan). The confirmed colonies were enumerated and CFU concentrations calculated per gram of dust.

Statistical analyses

Species (or assay groups) measured by QPCR at average concentrations of less than 2 spores per 5 mg of dust in either MH or RH were eliminated from further analysis. The remaining species or assay groups were each compared by calculating the ratio of the GM in MH to GM in RH. Assay results were categorized into those that had GM ratios >1.0 (Group I) and those <1.0 (Group II).

The differences in the concentrations of measured spores of the species or assay groups between MH and RH were analyzed for statistical significance using the Mann-Whitney U test (SPSS statistical package, version 10, SPSS inc. Chicago, IL). Logistic regression analysis was performed using SAS Proc Probit (SAS Institute Inc., Cary, NC) to estimate the probability of whether a home could be predicted to be a MH based on the sum of the logs of the concentration of species or assay groups.

Results

QPCR assay standard curves and control sample analyses

Standard curve parameter values for the QPCR assays used in this study are listed in Table 1. Calculated estimates of the

Table 2 Geometric Mean (GM) of concentrations of measured mold spores in moldy homes (MH) and reference homes (RH) and the ratios (MH : RH) of the GMs. Species with at least 2 cells on average in both MH and RH are indicated in bold. Group I (MH : RH >1) are underlined

Mold species and assay groups	MH[a] #/5 mg	RH[a] #/5 mg	Ratio MH : RH
Aspergillus caespitosus	0.33	0.32	
Aspergillus carbonarius	0	0	
Aspergillus candidus	0.78	1.68	
Aspergillus cervinus	0	0	
Aspergillus clavatus grp.[b]	0	0	
Aspergillus flavipes	0	0	
Aspergillus flavus grp.[c]	0.94	1.89	
Aspergillus fumigatus grp.[d]	3.4	1.02	
***Aspergillus niger* grp.**[e]	8.67	10.43	**0.83**
Aspergillus niveus	0	0	
***Aspergilus ochraceus* grp.**[f]	7.69	4.12	***1.87***
Aspergillus paradoxus	0	0	
Aspergillus parasiticus	0	0	
Aspergillus penicillioides	280	209	***1.34***
Aspergillus puniceus	0	0.29	
Aspergillus restrictus grp.[g]	2.05	1.93	
Aspergillus sclerotiorum	1.77	1.68	
Aspergillus sydowii	0.26	0.95	
Aspergillus tamari	0.30	1.29	
Aspergillus terreus	1.22	0.36	
Aspergillus unguis	21.6	12.4	***1.74***
Aspergillus ustus	0.77	1.64	
Aspergillus versicolor	7.25	2.9	***2.50***
Aspergillus wentii	0	0	
Emericella nidulans grp.[h]	0.74	1.46	
Emericalla variecolor	0	0	
***Eurotium* grp.**[i]	273	145	***1.89***
Penicillium atramentosum	3.50	1.32	
Penicillium brevicompactum	6.70	16.9	**0.40**
Penicillium canescens	0	0	
***Penicillium chrysogenum* svar.2**[j]	10	17.3	**0.60**
Penicillium citreonigrum	0	0	
Penicillium citrinum grp.[k]	0	0	
Penicillium digitatum	0.71	0.34	
Penicillium grp.1[l]	0.3	0	
Penicillium grp. 2[m]	0.5	1.6	
Penicillium coprophilum	0	0	
Penicillium corylophilum	0.53	198	
Penicillium decumbens	0	0	
Penicillium expansum	0	0	
Penicillium fellutanum grp[n]	0	0	
Penicillium glandicola	0	0	
Penicillium griseofulvum	0.22	0	
Penicillium implicatum	0	0.2	
Penicillium islandicum	0.38	0.37	
Penicillium italicum	0	0	
Penicillium melinii	0	0	
Penicillium miczynskii	0	0	
Penicillium olsonii	0.79	0.23	
Penicillium oxalicum	7.8	88.4	**0.09**
Penicillium purpurogenum	3.0	0.79	
Penicillium raistrickii	0	0	
Penicillium restrictum	0.47	0	
Penicillium roquefortii	1.8	0.23	
Penicillium sclerotiorum	1.7	2.9	
Penicillium simplicissimum grp.[o]	0.11	0	
***Penicillium spinulosum* grp.**[p]	10.60	11.6	**0.91**
Penicillium variabile	11.80	15.9	**0.74**
Paecilomyces variotii	3.30	9.0	**0.37**
Aureobasidium pullulans	4685	6332	**0.74**
Acremonium strictum	12.8	25.2	**0.51**
Alternaria alternata	168	303	**0.56**
Chaetomium globosum	2.3	3.48	**0.66**
***Cladosporium cladosporioides*- svar.1**	4804	7335	**0.65**
***Cladosporium cladosporioides*- svar. 2**	62.20	149	**0.42**
Cladosporium herbarum	107	211	**0.57**
Cladosporium sphaerospermum	123	93	***1.32***
Epicoccum nigrum	6047	11147	**0.54**
Memnoniella echinata	0	0	
***Mucor* and *Rhizopus* grp**[q]	107	111	**0.97**
Rhizopus stolonifer	1.75	2.03	
Scopulariopsis brevicaulis	2.70	4.7	**0.57**
Scopulariopsis chartarum	0.50	1.3	
Stachybotrys chartarum	1.6	1.09	
Trichoderma asperellum grp.[r]	0	0.2	
Trichoderma harzianum	0.83	0.33	
Trichoderma longibrachiatum grp.[s]	0.65	0.9	

Table 2 Geometric Mean (GM) of concentrations of measured mold spores in moldy homes (MH) and reference homes (RH) and the ratios (MH : RH) of the GMs. Species with at least 2 cells on average in both MH and RH are indicated in bold. Group I (MH : RH >1) are underlined (*Continued*)

Mold species and assay groups	MH[a] #/5 mg	RH[a] #/5 mg	Ratio MH : RH
***Trichoderma viride* grp.**[t]	3.4	6.4	**0.53**
Ulocladium atrum	0.15	0.19	
Ulocladium chartarum	0.15	0	
Ulocladium botrytis	1.46	0.9	
Wallemia sebi	127	129	**0.99**

[a] Geometric mean of measured numbers per 5 mg dust. [b] Includes *A. clavatus* and *A. giganteus*. [c] Includes *A. flavus* and *A. oryzae*. [d] Includes *A. fumigatus* and *Neosartorya fischeri*. [e] Includes *A. niger*, *A. foetidus* and *A. pheonicis*. [f] Includes *A. ochraceus* and *A. ostianus*. [g] Includes *A. restrictus*, *A. caesillus* and *A. conicus*. [h] Includes *E. nidulans*, *E. quadrilineata* and *E. rugulosa*. [i] Includes *E. amstelodami*, *E. chevalieri*, *E. herbariorum, E. rubrum* and *E. repens*. [j] Includes dominant subgroup of species. [k] Includes *P. citrinum*, *P. sartoryi* and *P. westlingi*. [l] Includes *P. aurantiogriseum*, *P. freii*, *P. hirsutum*, *P. polonicum, P. tricolor*, *P. verrucosum* and *P. viridicatum*. [m] Includes *P. crustosum, P. camembertii*, *P. commune*, *P. echinulatum* and *P. solitum*. [n] Includes *P. fellutanum* and *P. charlesii*. [o] Includes *P. simplicissimum* and *P. ochrocloron*. [p] Includes *P. spinulosum*, *P. glabrum*, *P. lividum*, *P. pupurescens* and *P. thomii*. [q] Includes *M. amphibiorum*, *M. circinelloides*, *M. hiemalis*, *M. indicus*, *M. mucedo*, *M. racemosus*, *M. ramosissimus*, *R. azygosporus*, *R. homothalicus*, *R. microsporus*, *R. oligosporus* and *R. oryzae*. [r] Includes *T. asperellum* and *T. hamatum*. [s] Includes *T. longibrachiatum* and *T. citrinoviride*. [t] Includes *T. viride*, *T. atroviride* and *T. koningii*.

amplification factors and extrapolated mean sensitivities of the *Stachybotrys*, *Aspergillus*, *Penicillium* and *Paecilomyces* assays have been previously reported.[7,9] Corresponding values for the other assays used for the first time in this study ranged from 1.81 to 2.00 for the amplification factors and from less than one to approximately four conidia per sample for the extrapolated mean sensitivities, based upon a normalized *G. candidum* reference assay C_T value of 17.7 for 2×10^6 conidia of this organism per sample (results not shown).

To monitor the precision of the QPCR measurements, a total of 29 positive control samples, containing $\sim 10^3$ or 10^4 conidia of each of the strains listed in Table 1 were extracted and subjected to analyses by the same panel of assays over the same time period as the dust samples. Using the pooled variance among all assay C_T values for these control samples and the overall correlation between target assay and reference assay C_T values as previously described,[9] the variance of ΔC_T was determined to be 2.49. This corresponded to a 95% occurrence range about the mean of approximately 40 to 250% for individual measurements. Analyses of 15 sets of no DNA template, negative control samples over the same time period, using the same panel of assays, consistently produced no signals ($C_T = 40$).

QPCR analyses, using the *G. candidum* reference assay, gave a mean C_T value of 20.16, SD = 1.11, for the 37 dust sample extracts, compared with a mean value of 18.90, SD = 1.05, for the 29 control sample extracts. Three of the dust sample reference assay C_T values were three standard deviations higher than the mean of the control sample results. However, further analyses of these samples provided no indications of matrix related PCR inhibition.[9] Based upon these results, the mean recovery of fungal DNA from the dust samples in the extraction process was 43% of the control samples and 20% of the normalized standard curve samples. This would indicate, on average, a five-fold reduction in the reported assays' sensitivities caused by the dust matrices. No significant difference was seen in the reference assay C_T values of the MH and RH samples ($P = 0.69$).

Differences between MH and RH in QPCR results

The average total concentrations of mold spores or conidia in MH and RH, as determined from the combined QPCR assay results, were 44 300 and 54 300 spores per 5 mg dust, respectively, and these concentrations were not significantly different ($P = 0.43$). There were also no significant differences in the concentration of any individual species or assay groups between MH and RH, nor was there a significant difference ($P = 0.57$) between the average number of different species found in MH (24.5) and in RH (25.5). There were 57 species or assay groups that were only rarely encountered (less than 2 spores per 5 mg dust on average) and these were eliminated from further analysis. Of the remaining species or groups, 6 had GM ratios (MH : RH) >1 and 19 had GM ratios (MH : RH) <1 (Table 2).

The sum of the logs of the concentrations of molds in the >1 category (Group I) was significantly higher in MH compared to RH ($P < 0.04$). Those molds with a GM ratio of <1 (Group II) were significantly lower in MH compared to RH ($P < 0.02$). The logistic regression analysis of the sum of the logs of the concentration of the 6 species in Group I gave a 95% probability of the house being categorized as MH, if the sum of the logs of the concentrations in Group I was found to be >19.4 (95% fiducial limits: 14.0 to 280.4).

Comparison of QPCR and culture based analyses

The concentrations of *Aspergillus fumigatus, A. niger, A. ochraceus* and *A. versicolor* that were calculated from standard culture-based methods (10^1 to 10^2 CFU g^{-1}) were three orders of magnitude lower than the concentrations of these same species as measured by QPCR analysis (10^3 to 10^5 cells g^{-1}) of the same dust samples (Fig. 1). When the QPCR and cultivation results were compared in the different samples, no significant correlation was found between these two techniques.

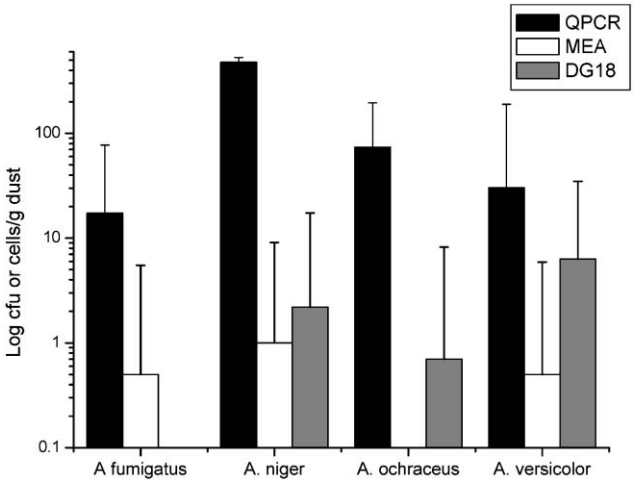

Fig. 1 Comparison of QPCR measurements and culture-based measurements on 2% malt extract agar (MEA) and dichloran-18-glycerol agar (DG18) of four *Aspergillus* species in dust samples from homes in Cincinnati, OH. Concentrations are presented as geometric means and geometric standard deviations.

Discussion

QPCR offers a standardized method for the identification and enumeration of molds. It is rapid and easy to perform. The QPCR assays employed in this study are, for the most part, species specific, however, in some cases several species with identical or nearly identical rDNA sequences are simultaneously measured (Table 2). If it becomes necessary to discriminate between these species, other genes will need to be targeted.[12,13] In a few cases, *e.g. P. chrysogenum* or *C. cladosporioides*, there are multiple rDNA sequevars that might eventually be separated as new species. The assay(s) used in this study detect the sequevar(s) most commonly found indoors.

Virtually all buildings contain molds that are normally introduced primarily from the outside environment.[14] The average total concentration of all molds measured by QPCR in this study was essentially the same in all house dust samples examined and no individual mold was statistically significantly different in MH and RH. Therefore, an empirical process was developed to categorize the molds relevant to distinguishing MH from RH.

The various mold species or assay groups were divided into categories based upon occurrence. Most of the species or groups (n = 57) were not common enough to be evaluated in this manner and were removed from the analysis. By taking the sum of the logs of the concentrations of the 6 species in Group I, abnormal mold conditions in a home, defined in this study as visible mold damage, can be distinguished from a home with no visible mold damage. If the sum of the logs of the concentrations of species in Group I is greater than 19.4, there is at least a 95% probability that it is a MH. The use of easily collected building dust samples in conjunction with this rapid form of analysis offers great potential advantages over other sampling and analysis procedures for identifying mold problems in buildings. For example, this type of analysis could allow for the relatively effortless and inexpensive presumptive identification of mold incursions in hidden building areas such as wall cavities without destructive surface sampling or long term air sampling.

While a large number of assays were employed in this exploratory investigation, the results suggest that analyses of only a few species or groups of species may be all that is necessary to establish that there is an abnormal mold condition. More comprehensive and extensive studies will be required to determine whether this kind of sampling, limited analysis and data handling can be used to describe the mold condition of homes or other buildings in other geographic regions and under all circumstances.

As presently practiced in the industry, the cultivation of the four *Aspergillus* species examined in this study did not accurately represent the concentrations of these molds in the dust samples. The viable spore concentrations, as measured by cultivation, were much lower than the total spore concentrations, as measured by QPCR. This trend has been previously reported when comparing results from cultivation analysis to those from total microscopic counting.[15] Whether viable or not, mold spores are still potentially allergenic and toxigenic.[16] If the relationship between mold exposure and health is going to be understood, the species composition and concentrations of these species, particularly in the indoor environment, must be accurately measured. The observation that culturing underestimated the concentrations of these four representative *Aspergillus* species suggests that accurate risk assessments can't be accurately made based on culture data.

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References

1 *Field guide for the determination of biological contaminants in environmental samples*, ed. H. K. Dillon, P. A. Heinsohn and D. Miller, American Industrial Hygiene Association, Fairfax, Virginia, 1996.
2 *Bioaerosols: Assessment and Control*, ed. J. Macher, American Conference of Governmental Industrial Hygienists, Cinncinnati, OH, USA, 1999.
3 G. L. Chew, C. Rogers, H. A. Burge, M. L. Muilenberg and D. R. Gold, *Allergy*, 2003, **58**, 13.
4 H. J. Chao, D. K. Milton, J. Schwartz and H. A. Burge, *Mycopathologia*, 2001, **154**, 93.
5 R. A. Haugland and S. J. Vesper, *Identification and Quantification of Specific Fungi and Bacteria: US Patent 6 387 652*, US Patent and Trademark Office, Washington, DC, 2002.
6 N. E. Brinkman, R. A. Haugland, L. J. Wymer, M. Byappanahalli, R. L. Whitman and S. J. Vesper, *Appl. Environ. Microbiol.*, 2003, **69**, 1775.
7 R. A. Haugland, S. J. Vesper and L. J. Wymer, *Mol. Cell. Probes*, 1999, **13**, 329.
8 R. A. Haugland, N. E. Brinkman and S. J. Vesper, *J. Microbiol. Methods*, 2002, **50**, 319.
9 R. A. Haugland, M. Varma, L. J. Wymer and S. J. Vesper, *Syst. Appl. Microbiol.*, 2004, **27**, 198.
10 J. Roe, R. A. Haugland, S. J. Vesper and L. J. Wymer, *J. Exposure Anal. Environ. Epidemiol.*, 2001, **11**, 12.
11 *Air Quality Monographs, Vol. 2. Health Implications of Fungi in Indoor Environments*, ed. R. A. Samson, B. Flannigan, M. E. Flannigan, A. P. Verhoeff, O. C. G. Adan and E. H. Hoekstra, Elsevier, Amsterdam, 1994, ch 7.
12 K. A. Seifert and G. Louis-Seize, in *Integration of Modern Taxonomic Methods for Penicillium and Aspergillus Classification*, ed. R. A. Samson and J. I. Pitt, Harwood Academic Publishers, Amsterdam, 2000, pp. 163–178.
13 M. Cruse, R. Telerant, T. Gallagher, T. Lee and J. Taylor, *Mycologia*, 2002, **94**, 814.
14 *Air Quality Monographs, Vol. 2: Health Implications of Fungi in Indoor Environments*, ed. R. A. Samson, B. Flannigan, M. E. Flannigan, A. P. Verhoeff, O. C. G. Adan and E. H. Hoekstra, Elsevier, Amsterdam, 1994, ch. 3.
15 M. Toivola, S. Alm, T. Reponen, S. Kolari and A. Nevalainen, *J. Environ. Monit.*, 2002, **4**, 166.
16 P. P. Kozak, Jr., J. Gallup, L. H. Cummins and S. A. Gillman, *Ann. Allergy*, 1980, **45**, 167.