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Determining Fungi rRNA Copy Number by PCR

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The goal of this project is to improve the quantification of indoor fungal pollutants via the specific application of quantitative PCR (qPCR). Improvement will be made in the controls used in current qPCR applications. This work focuses on the use of two separate controls within a standard qPCR reaction. The first control developed was the internal standard control gene, *benA*. This gene encodes for β-tubulin and was selected based on its single-copy nature. The second control developed was the standard control plasmid, which contained a fragment of the ribosomal RNA (rRNA) gene and produced a specific PCR product. The results confirm the multicopy nature of the rRNA region in several filamentous fungi and show that we can quantify fungi of unknown genome size over a range of spore extractions by inclusion of these two standard controls. Advances in qPCR have led to extremely sensitive and quantitative methods for single-copy genes; however, it has not been well established that the rRNA can be used to quantitate fungal contamination. We report on the use of qPCR, combined with two controls, to identify and quantify indoor fungal contaminants with a greater degree of confidence than has been achieved previously. Advances in indoor environmental health have demonstrated that contamination of the built environment by the filamentous fungi has adverse impacts on the health of building occupants. This study meets the need for more accurate and reliable methods for fungal identification and quantitation in the indoor environment.

KEY WORDS: qPCR, indoor environment, health, biocontaminants

INTRODUCTION

Improved methods of fungal identification and quantification are needed to gain a better understanding of fungal impacts in the indoor environment. This manuscript details the development of two internal controls for use in molecular applications used to quantify indoor fungal pollutants. In North America, it is estimated that people spend between 80% and 90% of their time in the indoor environment.[1,2] Thus, the importance of a healthy indoor environment has become increasingly significant in recent years. The predominant indoor allergens are from mites, cats, and dogs.[3] However, the indoor environment also contains a wide range of microorganisms, including bacteria and filamentous fungi.[4–6] Of these biological contaminants, the filamentous fungi (mold) are now recognized as key contributors to an unhealthy built environment, with up to 40% of North American and Northern European homes containing mold infestation.[7,8]

The filamentous fungi found in the indoor environment can produce microbial volatile organic compounds, allergenic proteins, and mycotoxins,[9–13] each contributing to adverse health effects, including itchy eyes, stuffy nose, headache, and general fatigue.[12,14–19] As such, the use of the phrase "sick building syndrome" is being applied more frequently when the possible cause is fungal contamination.[20]

Laboratory confirmation and quantitation of mold in the built environment typically include microscopy, morphology, and colony counting.[21–23] These methods of analysis are not only time-consuming, but they also frequently misidentify organisms and lead to an understatement of the diversity of the indoor fungal community.[24] These concerns have led to the rapid development of molecular technologies that are capable of unambiguous identification, as well as quantification of fungal contamination. At the forefront of these molecular

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tools is the qPCR. A favorite target for designing a qPCR assay is the repetitive and highly conserved nuclear rRNA region of fungi.[25–30] Recent advances in qPCR have led to extremely sensitive and quantitative methods for single-copy genes; however, it has not been well-established that the rRNA can be used to quantitate fungal contamination. The adherent problem when using this region for qPCR is that its multicopy nature is not defined and has been shown to number in the tens to hundreds for a few of the now fully sequenced fungi.[31,32]

MATERIALS AND METHODS

Filamentous Fungi, Spore Preparations, and DNA Isolation

The test organisms selected for this project were in-house field isolates *Alternaria alternata*, *Aspergillus fumigatus*, *Aspergillus versicolor*, *Cladosporium cladosporoides*, *Stachybotrys chartarum 5108*, *S. chartarum 5111*, *S. chartarum 6307*, and *S. chartarum Hous*, cultured for 14–21 days at room temperature under incandescent lights. Spores were collected, and DNA was extracted using a method described previously .[33]

Cloning of *C. cladosporoides* Insert

To estimate the rRNA gene copy number in filamentous fungi, an internal fragment of this gene was cloned. Enzyme restriction and PCR amplification of the rRNA region of *C. cladosporoides* (Accession AJ244241) produced a 450-bp product that was ligated into a pGEM-T Easy vector (Promega, Madison, WI, USA) and transformed into *Escherichia coli* strain DH5α. Bacteria were taken up in 800 μL LB broth medium and allowed to incubate for 45 min at 37°C and 225 rpm. After incubation, bacteria were plated onto agar plates containing ampicillin (100 μg/mL),

TABLE 1

Real-Time PCR Primers Used in This Study

Organism	Primer	Sequence (5′→3′)	Tm (°C)	Amplicon (bp)	Set point (°C)
PCR insert					
C. cladosporoides	Sense	ATC ATT ACA AGT GAC CCC GGC	60	450	60
	Antisense	TTT ACG GCG TAG CCT CCC	58		
rRNA					
A. alternata	Sense	CGA ATC TTT GAA CGC ACA TTG	57	163	57
	Antisense	CGC TCC GAA ACC AG TAG G	58		
A. fumigatus	Sense	CTG TCC GAG CGT CAT TGC	58	140	57
	Antisense	TAC AGA GCA GGT GAC AAA GC	58		
A. versicolor	Sense	GCG AAC TGC GAT AAG TAA TGT	57	111	57
	Antisense	GCA ATG ACG CTC GGA CAG	58		
C. cladosporoides	Sense	GCG TCA TTT CAC CAC TCA AG	58	128	57
	Antisense	CTC CCG AAC ACC CTT TAG	56		
Penicillium chrysogenum	Sense	CTG TCC GAG CGT CAT TGC	58	137	57
	Antisense	CAG AGC GGG TGA CAA AGC	58		
S. chartarum	Sense	GAG TAG TTT GGG AAT GCT GC	58	174	57
	Antisense	GCC CAA GTC TGG TCA TAA AC	58		
β-Tubulin					
A. alternata	Sense	GCG AAC AAC AGA GAA CAA CAG	54	143	55
	Antisense	AAA GGC AGC ACC GAT TTG G	56		
A. fumigatus	Sense	GCT GGA GCG TAT GAA CGT C	56	165	55
	Antisense	GAC AGC GTC CAT GGT ACC	55		
A. versicolor	Sense	GCG AAC TGC GAT AAG TAA TGT	55	108	55
	Antisense	GCA ATG ACG CTC GGA CAG	56		
C. cladosporoides	Sense	GCA TAC ACC GAT TGA CAA C GCC	55	121	55
	Antisense	TTG GGA GTT CAT ACC	56		
P. chrysogenum	Sense	CGA TGG CGA TGG ACA GTA AG	55	131	55
	Antisense	ACT CAC ATG GTT GAA GTA GAC G	55		
S. chartarum	Sense	ACC TCT CGG CTC ACA ATT TC	55	165	55
	Antisense	TTG TTG CCA GCA CCA GAC	56		

Tm, Temperature.

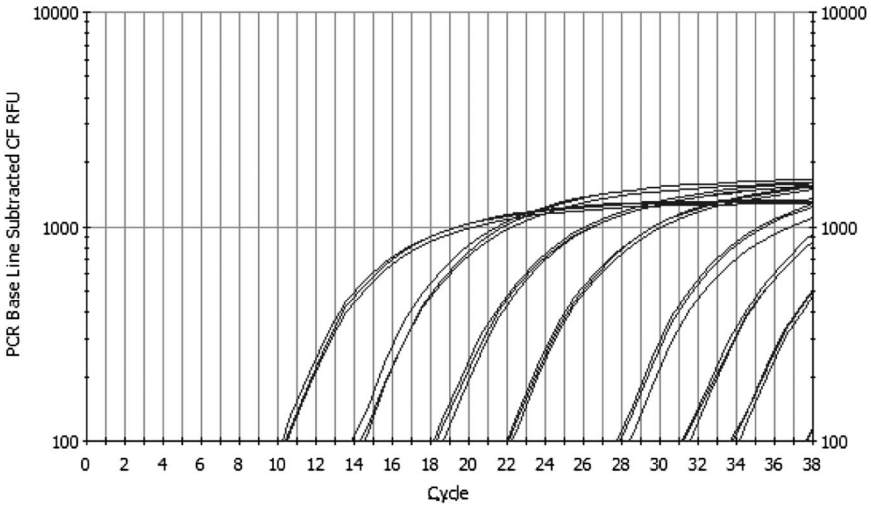

FIGURE 1

Real-time PCR of p57 internal standard. Copy number profile of the p57 plasmid containing the 450-bp *C. cladosporoides* (Cc) insert was determined by qPCR. The first curve set is 10^6, tenfold serially diluted to 10^0. The copy number was determined by mass, and correlation coefficient of the curve is 0.996 (experiment was run in triplicate).

5-bromo-4-chloro-3-indolyl-β-D-galactoside (20 μg/cm^2), and IPTG (12.1 μg/cm^2) and incubated overnight at 37°C. White colonies were selected and the inserted gene fragment confirmed by PCR using Cladc F1/R1 primers (Table 1). Recombinant plasmid DNA was purified using an UltraClean 6 Minute Mini Plasmid Prep Kit (MO BIO Laboratories, Carlsbad, CA, USA). After purification, plasmid DNA concentration was determined using a Quant-iT dsDNA high-sensitivity assay kit and Qubit fluorometer (Invitrogen, Eugene, OR, USA). We determined that the plasmid (p57) contained a single-copy insert by agarose gel electrophoresis (data not shown). The copy number was adjusted to 10^7 in Tris-HCl buffer.

Real-Time PCR

Primers used for this study are presented in Table 1. They were synthesized and High Purity Salt Free (HPSF)-purified by MWG-Biotech AG, VWR International (Bridgeport, NJ, USA). qPCR reactions were performed in triplicate and comprised genomic DNA, combined forward and reverse primers (1.2 μM), iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA), and water to 25 μL. The reactions were placed in a Bio-Rad iCycler (Bio-Rad Laboratories). The iCycler was programmed to run on gradient function with annealing temperatures of 60°C, 57°C, and 55°C for each primer set (Table 1). Samples were denatured at 95°C for 4 min, followed by 40 cycles of 95°C (10 s), annealed (10 s), and 72°C (10 s). The relative concentration of the amplified DNA was calculated using the comparative quantification feature in the iCycler software package against the relative fluorescence signal.

RESULTS

The coding sequences were obtained for each gene after an exhaustive search of the PubMed database. In all cases, multiple sequences for each gene were selected and aligned as to design internal oligonucleotide primers for the applications in the qPCR as described in Materials and Methods. Each qPCR assay was first optimized for each primer set by varying primer and PCR reagent concentrations to obtain peak efficiencies (data not shown). The specificity of each primer set to amplify the target was confirmed by melt curve analysis.

Real-time PCR analysis of the p57 standard control plasmid produced specific PCR products that were amplified using primer set Cc insert (Fig. 1) The tenfold serial difference in template concentrations was used to test the sensitivity of a real-time PCR assay. Plasmid DNA was run in triplicate, and comparative threshold (C_t) values ranged from 10 (10^6 copies) to 34 (10^0 copies). The C_t values obtained between each log dilution were between three and four in almost all cases. Overall, the curve generated from the p57 qPCR proved to be nearly linear with a correlation coefficient (r) of 0.996. Based on these results, we believed that p57 could be used to determine the absolute copy number of the rRNA region in filamentous fungi. However, as we do not know the size of the genome for each of the fungi used in this study, and we did not know the efficiency of the genomic DNA extraction from a spore, we also included a genomic internal standard control.

The genomic internal standard control gene, *benA*, was selected based on its single-copy nature and encodes for β-tubulin. When we conducted qPCR on genomic preparations, we found that the rRNA gene was, in all cases, at least 2 log higher in copy number than the *benA* gene (Fig. 2).

The curves demonstrate the relationship of each target region over a 5-log genomic DNA extraction of spores. In general, there was an ~2-log difference in copy number between the multicopy rRNA and single-copy β-tubulin region, and the results were reproducible. Detection of all fungi was optimal when as few as 10^4 spores were processed

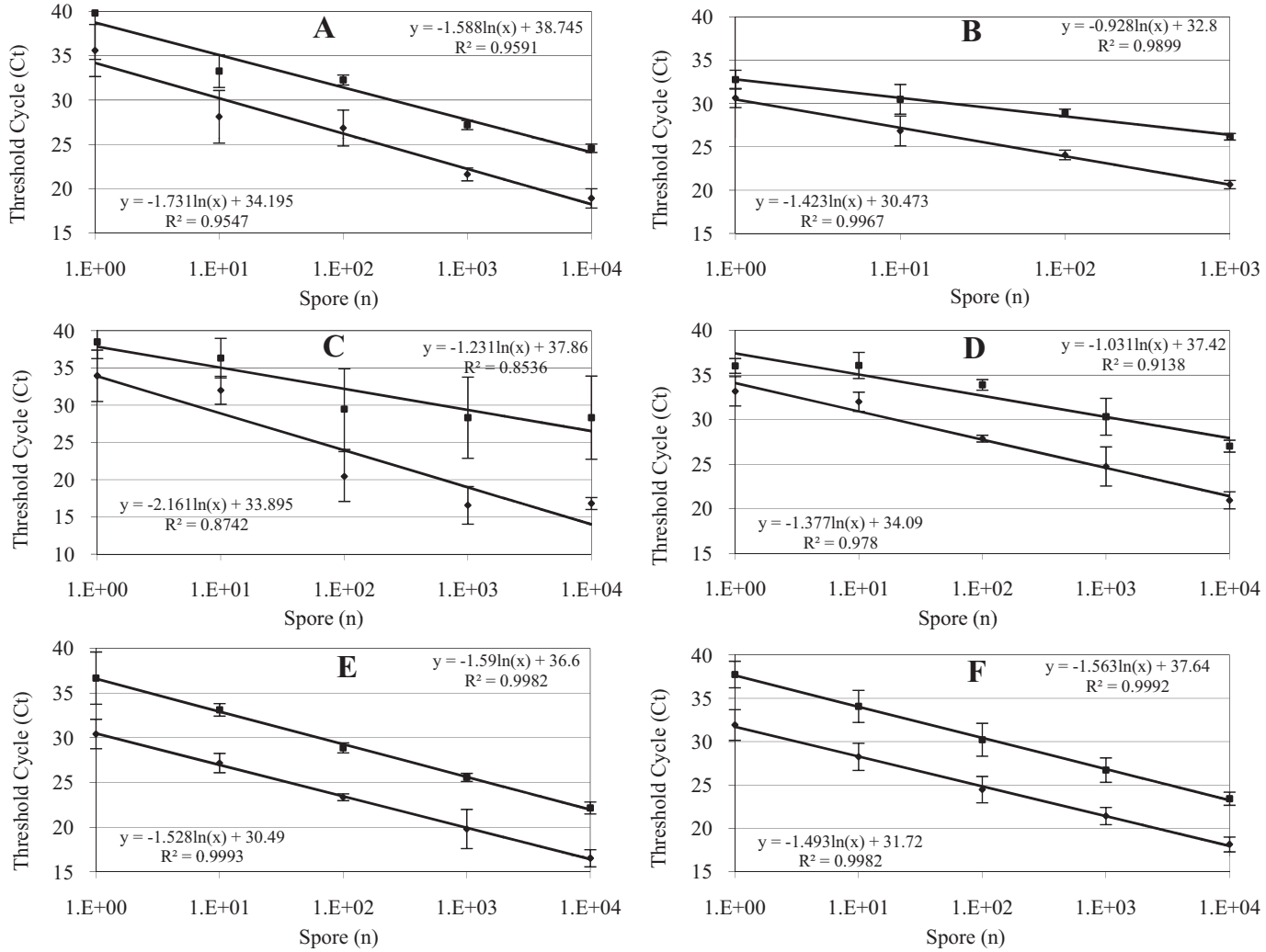

FIGURE 2

Real-time qPCR results of C_t versus number of spores (n) from which the template gDNA was extracted. *A. alternaria* (A), *A. fumigatus* (B), *C. cladospooides* (C), *P. chrysogenum* (D), *S. chartarum 5108* (E), and *S. chartarum 5111* (F). Each plot represents an average of three independent experiments amplifying rRNA (lower curves) and β-tubulin (upper curves). *r* was calculated for each plot.

but was also sensitive enough to detect as few as 10 spores in a single preparation. The C_t were similar, and detection began after 15 cycles or so for rRNA and 20 cycles or so for β-tubulin. Linear regression equations with *r* values >0.95 were obtained in all experiments except for *C. cladosporoides* (0.85–0.87).

Each C_t value is the result of fluorescence produced by SYBR Green I excitation during the annealing step. Each experiment was done in triplicate, and SEM is included. The data indicate that although absolute copy number cannot be predicted from this type of experiment alone, the difference in copy number can be predicted by this qPCR. This is true even when the genome size of the fungi is not known. We observed that there was almost a six C_t difference between the two genes over the range of extractions with the exception of *C. cladosporoides*. From the data gathered in Fig. 1, we conclude that a C_t value of three to four indicates a 1-log difference. As such, a six C_t is indicative of ten- to 100-fold differences in copy number. The ΔC_t remains consistent over the qPCR range but does vary when few copies of the target are analyzed.

In a final experiment, we conducted qPCR using p57 as the standard control curve. The p57 was prepared using mass as the method to determine copy number. In this case, the 3465-bp plasmid is calculated to weigh 3.80E–18 g. Using this as a starting point, we determined the amount of p57 equivalent to 10^6, 10^4, 10^2, and 10^1 copies for a near-linear curve with an *r* of 0.997. However, because the genome sizes are unknown, the same calculations cannot be made for the fungi. Based on our previous data, we selected

TABLE 2

qPCR and Prediction of Gene Copy Number in Fungi

Target	Identifier	Quantity	Log-starting quantity	Quantity (calculated)
rRNA	*A. alternata*	1.00E + 03	2.97	9.32E + 02
	A. fumigatus	1.00E + 03	3.14	1.36E + 03
	A. versicolor	1.00E + 03	2.77	5.88E + 02
	C. cladosporoides	1.00E + 03	3.40	2.50E + 03
	S. chartarum 5108	1.00E + 03	3.50	3.10E + 03
	S. chartarum 5111	1.00E + 03	3.01	1.03E + 03
	S. chartarum 6307	1.00E + 03	3.75	5.66E + 03
	S. chartarum Hous	1.00E + 03	3.38	2.38E + 03
β-Tub	*A. alternata*	1.00E + 03	1.51	3.25E + 01
	A. fumigatus	1.00E + 03	1.71	5.09E + 01
	A. versicolor	1.00E + 03	1.25	1.79E + 01
	C. cladosporoides	1.00E + 03	−1.08	8.33E − 02
	S. chartarum 5108	1.00E + 03	1.25	1.77E + 01
	S. chartarum 5111	1.00E + 03	0.92	8.31E + 00
	S. chartarum 6307	1.00E + 03	0.97	9.35E + 00
	S. chartarum Hous	1.00E + 03	1.45	2.82E + 01

10^3 spores for this qPCR analysis. The log concentration of each fungi DNA product was calculated relative to the p57 standard curve using the comparative quantification feature in the iCycler software package against the relative fluorescence signal (Table 2).

Our data indicate that the rRNA multicopy region of 10^3 fungi has a copy number lower than expected. For example, if the rRNA copy number is 100 or more than the starting quantity of *A. alternata*, then there should be 1.00E + 05 or more for 10^3 spores, and the single-copy gene *benA* should be 1.00E + 03. Here, the copy number is reported as 9.32E + 02 and 3.25E + 01, respectively. Both quantities are ~2 logs less than expected. In general, this is what was observed for all of the fungi in this study. As the p57 was used as mass, and the standard curve of p57 was nearly linear (r=0.997), we conclude that the extraction of the spores is inefficient. We believe this illustrates the importance of using actual spore quantities for each genomic DNA extraction rather than dilution of a single high-spore preparation.

DISCUSSION

The curves and copy number data produced from our experiments show that qPCR can unequivocally confirm the multicopy nature of the rRNA region in several filamentous fungi over a range of spore extractions. Determination of gene copy number is usually correlated to a known genome size, which in fungi, can vary between 15.8 and 49 Mbp[34–45] and an average genome size of 36 Mbp.[46] We demonstrate that qPCR can be used for quantitation of these fungi, even when genome size is not known. We accomplished this by inclusion of two standard controls. The first, generated by our laboratory, is a plasmid-containing fungi rRNA that could be quantified by mass as to exact copy number in the qPCR, whereas the second is a single-copy β-tubulin (*benA*) gene and served as an internal standard of the genomic DNA preparation.

This study also illustrates the difficulty in determining organism copy number by qPCR alone, as has been reported previously for other microbes, including bacteria, yeast, and fungi.[47–52] This and these previous studies found between 20% and 50% variation in the detectable target DNA when starting quantity was known. However, these data do not invalidate the approach; they only highlights the necessity for better genomic DNA isolation procedures when dealing with spore and spore-like structures.

As a result of the need for more accurate and reliable methods for fungal identification and quantitation, we set out to generate experimental assay controls that could enhance currently used qPCR methodologies. To that end, we have developed two controls that can be used to better quantify fungal organisms from environmental samples. Additionally, our research has drawn emphasis on the need for more efficient fungal DNA isolation and purification procedures.

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