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Evaluation of different methods for the extraction of DNA from fungal conidia by quantitative competitive PCR analysis

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Abstract

Five different DNA extraction methods were evaluated for their effectiveness in recovering PCR templates from the conidia of a series of fungal species often encountered in indoor air. The test organisms were *Aspergillus versicolor*, *Penicillium chrysogenum*, *Stachybotrys chartarum*, *Cladosporium herbarum* and *Alternaria alternata*. The extraction methods differed in their use of different cell lysis procedures. These included grinding in liquid nitrogen, grinding at ambient temperature, sonication, glass bead milling and freeze–thawing. DNA purification and recovery from the lysates were performed using a commercially available system based on the selective binding of nucleic acids to glass milk. A simple quantitative competitive polymerase chain reaction (QC-PCR) assay was developed for use in determining copy numbers of the internal transcribed spacer (ITS) regions of the ribosomal RNA operon (rDNA) in the total DNA extracts. These quantitative analyses demonstrated that the method using glass bead milling was most effective in recovering PCR templates from each of the different types of conidia both in terms of absolute copy numbers recovered and also in terms of lowest extract to extract variability. Calculations of average template copy yield per conidium in this study indicate that the bead milling method is sufficient to support the detection of less than ten conidia of each of the different organisms in a PCR assay.

Keywords: DNA extraction; Fungal conidia; Quantitative competitive PCR; Ribosomal DNA

1. Introduction

Interest in the fungal composition of indoor air has increased greatly in recent decades in association with a growing awareness of the potential adverse health effects caused by these organisms and the overall significance of indoor air quality on human health (Tobin et al., 1987; Flannigan et al., 1991; Flannigan and Miller, 1994; Husman, 1996; Verhoeff and Burge, 1997). While the importance of both qualitative and quantitative determinations of indoor air spora has been repeatedly stressed, methods for the identification and enumeration of different fungal species in air samples have not improved significantly over the years. These methods, for the most part, still rely on the time consuming and labor intensive practices of plating the samples on growth media and inspecting the resultant colonies microscopically for morphological characteristics (Solomon, 1976; Hunter et al., 1988; Yang et al.,

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1993). The polymerase chain reaction (PCR) is increasingly being used as an alternative to culture-based methods for the detection and, in some cases, quantitation of microorganisms in clinical, food and environmental samples (Persing et al., 1993; Zimmermann and Mannhalter, 1996; Pillai, 1997). Recent technological advances allowing direct detection of PCR products in the reactions (Livak et al., 1995; Bassler et al., 1995) and greater speed and accuracy in the quantitative measurements of target sequences (Heid et al., 1996) portend even more extensive use of this method in the future. Our laboratory is currently investigating the potential application of this technology for the monitoring of conidia from certain species of toxigenic and allergenic fungi in indoor air (Haugland and Heckman, 1998).

An important issue that must be considered in determining the sensitivity and precision of PCR-based methods for the detection of microorganisms is the efficiency of the cell lysis and nucleic acid purification methods used to recover DNA or RNA templates (Wilson, 1997). The well-known resistance of fungal spores and conidia to lysis as well as the potential presence of inhibitory compounds in these cells (Martin and Haider, 1971) could significantly impair the sensitivity of a PCR method designed for their detection. Several studies have been devoted to the development and/or comparison of different procedures for the extraction of DNA from fungal and bacterial spores or conidia (Moller et al., 1992; Gang and Weber, 1995; Kutchma et al., 1998; Muller et al., 1998; Kuske et al., 1998). These studies, however, have generally utilized much higher numbers of cells than would normally be collected in air samples (Solomon, 1976; Hunter et al., 1988; Yang et al., 1993) and have quantified their yields in terms of the total amounts of DNA in the extracts. At best, such measurements allow only approximate estimates to be made of the sensitivity of target organism detection that might be supported in a subsequent PCR assay.

In the current study, several rapid and simple cell lysis procedures, used in conjunction with a commercially available DNA purification kit, were evaluated for their effectiveness in recovering DNA from the conidia of diverse airborne fungal species including *Aspergillus versicolor*, *Penicillium chrysogenum*, *Cladosporium herbarum*, *Stachybotrys chartarum* and *Alternaria alternata*. In an effort to approximate the overall quantities of cells that might be collected in air samples in association with a PCR analytical method, extractions were performed on 10^4 or 10^5 conidia in each of these experiments. The DNA yields in the extracts were determined using a PCR approach that targeted a segment of the ribosomal RNA operon (rDNA) containing the internal transcribed spacer (ITS) regions and 5.8S rRNA gene. A quantitative competitive PCR (QC-PCR) assay using corresponding templates from a designated non-target fungal species as standards was developed and validated and then used to obtain quantitative measurements of rDNA sequences in the extracts. In addition to allowing a comparison of the effectiveness of the different DNA extraction methods, the results from this study provide a preliminary indication of the sensitivity and precision of conidial detection that might be attainable for these species in a PCR-based air analysis method.

2. Materials and methods

2.1. Fungal cultures and conidial stocks

The fungal strains used in this study and their sources are listed in Table 1. Cultures were routinely grown for 7–12 days at room temperature on Czapek's yeast agar medium (Pitt, 1973) for the preparation of conidial stocks. The conidia were harvested by gently rolling a moistened, sterile cotton swab over the surface of the colonies and then suspended in 0.05% Tween 80. After counting with a hemocytometer, the conidial stock suspensions were adjusted to a concentration of either approximately 10^6 (for *Alternaria*) or 10^7 (for all other species) cells/ml, aliquoted and stored at -80°C.

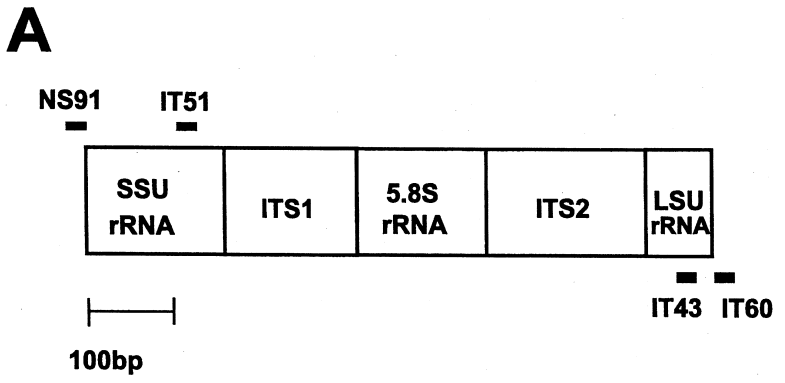
2.2. QC-PCR using purified rDNA templates

DNA templates in these experiments were PCR amplicons generated using the primers NS91 and IT60 (Fig. 1). Methods for the preparation, purification and copy number quantitation of these amplicons have been previously described (Haugland and Heckman, 1998). Serial dilutions of template

Table 1
Fungal cultures and conidial characteristics

Species, EPA collection number	Source, strain	Conidial shape	Approximate conidial size range (μm^3)
Aspergillus versicolor, 370	SRC[a], 153	Oval	5–15
Penicillium chrysogenum, 309	FRR[b], 807	Oval	5–35
Stachybotrys chartarum, 388	UAMH[c], 6417	Ellipsoid	100–450
Cladosporium herbarum, 380	UAMH, 7708	Ellipsoid	150–600
Alternaria alternata, 402	UAMH, 7707	Fusiform	800–5000[e]
Trychophyton rubrum, 218	ATCC[d], 28188	–	–

[a] USDA, Agricultural Research Service, Southern Regional Research Center.
[b] Commonwealth Scientific and Industrial Research Organization, Food Research Laboratory, North Ryde, NSW Australia.
[c] University of Alberta Microfungus Collection and Herbarium.
[d] American Type Culture Collection.
[e] Multicellular, average five cells per conidium.



B

PCR Primers:

NS91	5'-GTCCCTGCCCTTTGTACACAC-3'
IT51	5'-GAGGAAGTAAAAGTCGTAACAAGGT-3'
IT43	5'-CTTTTCCTCCGCTTATTGATATGC-3"
IT60	5'-CCGCTTCACTCGCCGTTACT-3'

Fig. 1. Map of the rDNA regions amplified in this study and features of the PCR primers used. (A) Target sites of the primers on the rDNA map. Abbreviations: SSU, small subunit; LSU, large subunit; ITS, internal transcribed spacer. Indicated ITS1 and ITS2 region lengths correspond to those of *Stachybotrys chartarum*. Forward primers are shown above the map and reverse primers are shown below. (B) Primer sequences. All primers were made on a Model 381A automated DNA synthesizer (PE Applied Biosystems, Foster City, CA, USA) and their concentrations determined on the basis of UV absorbance at 260 and 280 nm.

stock solutions were performed in distilled water immediately prior to each QC-PCR experiment. PCRs each contained in a total volume of 50 μl: 10 μl each of appropriately diluted target and competitor templates; 200 μM dNTPs; 500 nM each of IT51 and IT43 primers (Fig. 1); 1.5 mM $MgCl_2$; 0.2 mg/ml bovine serum albumin (fraction V, Sigma, St. Louis, MO, USA); 0.9 units Expand high fidelity PCR system (a mixture of Taq and Pwo DNA polymerases, Boerhinger Mannheim, Indianapolis, IN) and the PCR buffer provided with the polymerases. The reagents were overlayed with mineral oil and amplification was performed in a model 480 thermal cycler (Perkin-Elmer, Norwalk, CT, USA) for 25 step cycles consisting of 30 s at 96°C, 15 s at 50°C and 4 min at 68°C.

Analyses were routinely performed with fixed quantities of target templates and three different quantities of competitor templates (Fig. 2). Aliquots of the PCRs (10 μl) were subjected to electrophoresis in 1.5% agarose gels for 2 h at ~3.5 V/cm in standard Tris–Borate–EDTA buffer. The resolved amplicon DNA bands were visualized by staining with SYBR Green I (FMC, Rockland, ME, USA), and their fluorescence signals were measured with a model SI Fluorimager (Molecular Dynamics, Sunnyvale, CA, USA) using the associated software program, IMAGEQUANT. The measured signal values were normalized on the basis of the length differences of the target and competitor amplicons and ratios of the normalized values (Tn/Cn) were determined for each reaction. Logarithms of these ratios were plotted against the logarithms of the added copy numbers of competitor templates (Co) in each analysis. The logarithms of experimentally determined test template quantities in the reactions (To) were obtained by interpolation on the lines of best fit generated from these plots for the log Co values where log Tn/Cn equaled zero, i.e. the molar equivalence points between target and competitor PCR products (Cross, 1995).

2.3. Cell lysis and DNA purification procedures

Details of each of the cell lysis procedures used in this study are described below. In all instances, frozen conidial stocks were thawed, the conidia were thoroughly resuspended by vortexing and 10-μl aliquots of the suspensions (~10^4 Alternaria conidia, ~10^5 conidia of all other species) were taken for further treatment. Unless otherwise specified, all samples were purified after cell lysis with an Elu-Quik^R DNA purification kit (Schleicher and Schuell, Keene, NH, USA) using a modification of the manufacturers protocol. In this modified protocol, lysate–glass milk mixtures were incubated with constant tube inversion for 10 min and then transferred to SPIN filter and catch tube assemblies (BIO 101, Vista, CA, USA) for centrifugation at 7500 *g* for 1.5 min. The glass milk particles, with adsorbed nucleic acids, were washed twice in the filter cartridges by gentle vortexing with 0.5 ml of Elu-Quik^R wash buffer and once with 0.5 ml Elu-Quik^R salt reduction buffer and centrifuged as indicated above after each wash. Nucleic acids were released from the glass particles by vortexing sequentially with two 100-μl aliquots of distilled water and collected by centrifuging the eluates into clean catch tubes as described above. DNA samples obtained in this manner were stored at 4°C for subsequent PCR analysis.

2.3.1. Freeze–thaw lysis procedure

Conidial suspensions were mixed by vortexing with 100 μl of lysis buffer, 300 μl of binding buffer and 25 μl of glass concentrate from an Elu-Quik^R DNA purification kit and subjected to DNA purification as described above. No additional cell lysis treatments other than the initial thawing of the previously frozen conidial suspensions described above were employed in this procedure.

2.3.2. Sonication lysis procedure

Two hundred milligrams of 212–300 μm acid washed glass beads (Sigma, St. Louis, MO, USA) were weighed into 2-ml semi-conical screw cap polypropylene microfuge tubes. To these tubes, the Elu-Quik^R DNA purification kit reagents described in Section 2.3.1 were added together with conidial suspensions. After mixing the samples by inversion, the tubes were partially immersed in the water bath of an ultrasonic cleaning instrument (Branson Clean-

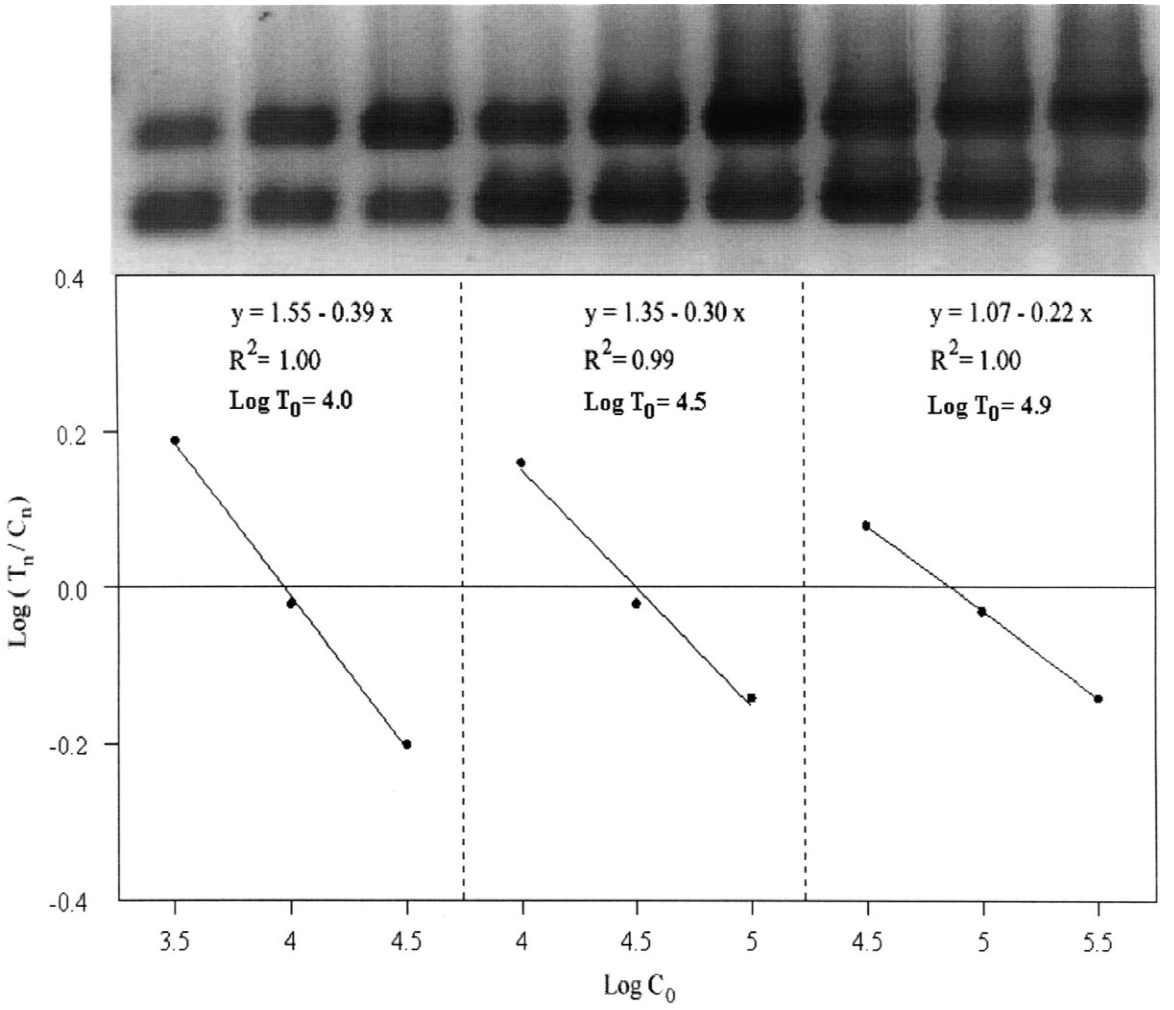


Fig. 2. Representative results illustrating the methodology used to experimentally quantify known copy numbers of purified test organism rDNA templates by QC-PCR. Upper panel: Competitor amplicons (upper bands) and test amplicons (lower bands) from three sets of three PCRs were resolved by agarose gel electrophoresis and visualized with a fluorescence imager after staining the gel with SYBR Green I. Each of the three reactions in a set was initiated with a fixed quantity of test templates but contained different quantities of competitors. Lanes 1–3 contain samples from PCRs initiated with 10^4 test templates and 3.3×10^3, 1×10^4 and 3.3×10^4 competitor templates. Lanes 4–6 contain samples from PCRs with 3.3×10^4 test templates and 1×10^4, 3.3×10^4 and 1×10^5 competitor templates. Lanes 7–9 contain samples from PCRs with 10^5 test templates and 3.3×10^4, 1×10^5 and 3.3×10^5 competitors. Fluorescence signal intensities of the bands were quantified by the imaging instrument and associated software. Lower panel: The length-normalized and $\log_{10}$-transformed ratios of the test and competitor amplicon signals (Tn/Cn) from each gel lane were plotted against the corresponding $\log_{10}$-transformed quantities of competitor templates (Co) in the reaction. Interpolation on the line of best fit from each of these plots for log Co values where log(Tn/Cn) equals zero provides a measure of the quantities of test organism templates (To) in that set of reactions. The test templates in these experiments were from *Pencillium chrysogenum*.

ing Equipment, Shelton, CT, USA). Sonication was performed at room temperature for 5 min according to the instrument manufacturers instructions.

2.3.3. Glass bead milling procedure

Acid washed glass beads and Elu-Quik kit components were mixed with conidial suspensions as

described in Section 2.3.2. The samples were milled with a Mini-Bead Beater (BioSpec Products, Bartlesville, OK, USA) for 1 min at 5000 reciprocations/min.

2.3.4. Ambient temperature grinding procedure

Conidial suspensions were transferred to 1.5-ml polypropylene tubes specially manufactured for use with a reusable CTFE polymeric head pellet pestle (Kontes, Vineland, NJ, USA). The suspensions were ground for 3 min at ~300 rpm with the pellet pestles driven by a variable speed homogenizer (Glas-Col, Terre Haute, IN, USA). The pestles were rinsed into the sample tubes with 100 and 300 μl of Elu-Quik^{R} lysis and binding buffers, respectively, and the samples mixed with 25 μl of Elu-Quik^{R} glass milk concentrate.

2.3.5. Liquid nitrogen grinding procedure

Conidial suspensions were transfered to pellet pestle tubes as described in Section 2.3.4. The tubes were plugged with sterile cotton and the samples were dried in a vacuum centrifuge (Savant Instruments, Holbrook, NY, USA). The tubes were then placed in a liquid nitrogen bath and the conidial pellets were ground with pellet pestles as described in Section 2.3.4. Unless otherwise specified in the text, the ground samples were suspended with Elu-Quik^{R} reagents as described in Section 2.3.4.

2.4. QC-PCR using total DNA extracts from conidia

PCRs were set up and performed as described in Section 2.2 with the exception that aliquots of DNA extracts were added in place of purified target templates. In most instances 5 μl of the extracts were used in these reactions, however, in some instances as little as 0.5 μl or as much as 10 μl of the extracts were used in order to obtain target PCR product yields within the range of the competitor product yields. Analyses of each extract were performed with four different quantities of competitor templates as illustrated in Fig. 3. Measurements of PCR product signals and determinations of log To were made as described in Section 2.2. Log To values were converted to template copy numbers in the total extracts based on the portions of the extracts used for each PCR. These values were converted to template copies recovered per conidium based on the numbers of conidia used in the extractions.

2.5. Statistical analyses

Recovered template copy number data were transformed to $\log_{10}$ values prior to analysis in order to achieve homogeneity of variance among the samples. Data transformed in this manner were shown to be reasonably normal in distribution ($P > 0.20$) by the method of Shapiro and Wilk (1968) for independent samples. SAS/STAT, v. 6.12 (SAS Institute, Cary, NC, USA) was used for all statistical calculations. Pairwise comparisons among individual members, either in total or by species or by conidial size, were controlled at an experimental error rate ($\alpha = 0.05$) with the use of Bonferroni adjusted P values (Snedecor and Cochran, 1980).

Relative precision among the five methods was evaluated via analysis of the coefficient of variation (CV) in template copies obtained by each method and species combination. A nonparametric procedure for multiple comparisons based on signed ranks (Hollander and Wolfe, 1973) was used to determine significant differences in CV values among the five methods.

3. Results and discussion

3.1. Development and testing of a QC-PCR assay for rDNA-ITS region sequences

The ITS1and ITS2 rDNA regions represent attractive targets for PCR-based fungal detection methods. Numerous studies have revealed that even closely related fungal species contain sequence differences in these regions that can be exploited for specific detection of these organisms using sequence specific PCR primers or probes (Haugland and Heckman, 1998; Gardes et al., 1991; Lee et al., 1993). In addition, the multicopy nature of rDNA in most fungal genomes (Clare et al., 1986; Garber et al., 1988; Howlett et al., 1997) should increase the sensitivity at which these organisms can be detected in such a method. Because of our laboratory's interest in targeting these rDNA regions for the

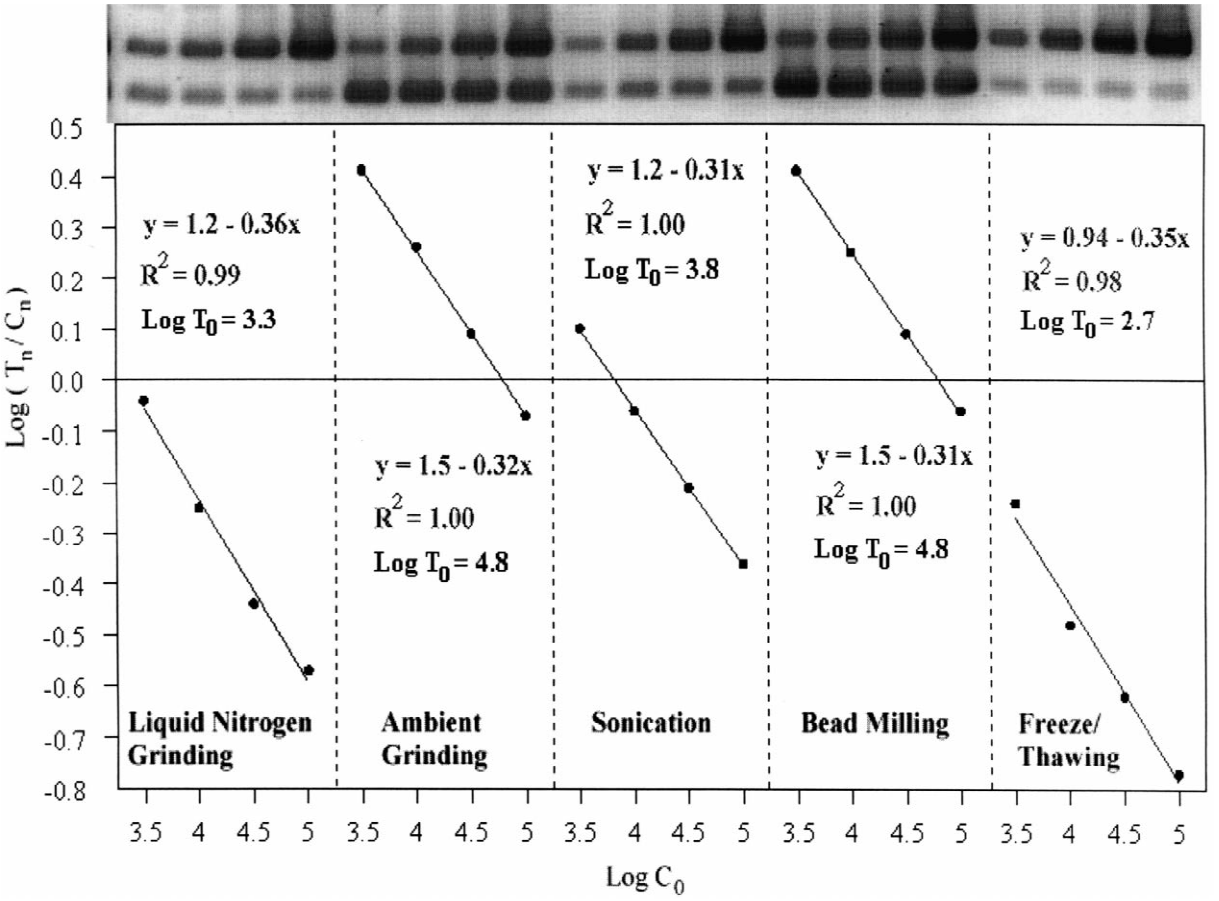


Fig. 3. Representative results illustrating the methodology used to quantify rDNA templates in DNA extracts obtained from conidia using different cell lysis procedures. In the experiments shown, all PCR reactions were initiated with 5 μl of DNA extracts. Quantities of competitor templates in each set of reactions were 3.3×10^3, 1×10^4, 3.3×10^4 and 1×10^5. Resolution and fluorescent image analysis of the test and competitor amplicons and manipulations of the data to determine the quantities of test organism templates (To) in the reactions were performed as described in the legend to Fig. 2 and Section 2.2 of the text. The DNA extracts in each of these experiments were from 10^5 conidia of *Cladosporium herbarum*.

development of PCR detection assays for a number of different fungal species with indoor health implications, it was desirable to evaluate the different DNA extraction methods examined in this study specifically in terms of their yields of these sequences. This required the development of an assay for quantifying these sequences in the DNA extracts that would be generally applicable for each of the test organisms.

QC-PCR is based on the coamplification of target sequences with different quantities of an appropriate internal standard template and has been the most widely used of all PCR quantitation methods (Zimmermann and Mannhalter, 1996). Several requirements must be met for accurate sequence quantitation in these assays. First, the target and competitor templates must contain identical or highly similar primer recognition sites. Second, the PCR products from target and competitor templates must be distinguishable in some manner, most commonly by length, using gel electrophoresis. Finally, and most importantly, the target and competitor sequences must be shown to amplify with equal efficiencies.

Data from the literature have indicated that certain small and large subunit rDNA sequences near the

ITS regions, such as those corresponding to the primers IT51 and IT43 (Fig. 1), are highly conserved in most eukaryotic species including fungi (White et al., 1990; Maidek et al., 1997). Unpublished results from our laboratory have shown that the IT51 and IT43 sequences are fully conserved in each of the species under investigation in this study. Numerous studies have also shown that the overall lengths of the ITS regions can vary substantially between different fungal species. Consequently, the possibility was considered of developing a simple QC-PCR assay using a PCR amplicon spanning the IT51 and IT43 primer recognition sites and ITS regions from an arbitrarily selected non-target fungal species as the competitor template. The template chosen for this purpose was from *Trichophyton rubrum* which produces a 722 bp IT51-IT43 PCR amplicon in comparison to the corresponding amplicons of the test species which range in length from 580 to 615 bp (data not shown).

In contrast to the majority of QC-PCR assays that have been reported, where competitor templates either have been selected or designed to be highly similar in sequence to the target templates, the proposed assay in this investigation incorporated a highly heterologous competitive template. For this reason, a series of preliminary experiments was performed to determine whether ITS region templates from the different test organisms were amplified by the IT51-IT43 primer pair at the same or at least similar efficiencies as the *T. rubrum* competitor template. The question was addressed by using known copy numbers of test organism templates in the QC-PCR assays and looking for agreement between these quantities and the quantities of the sequences determined in the assays. The results of these experiments (Fig. 4) revealed no significant differences between the templates added and the means of the measured quantities of any of the test organisms templates over the entire examined range

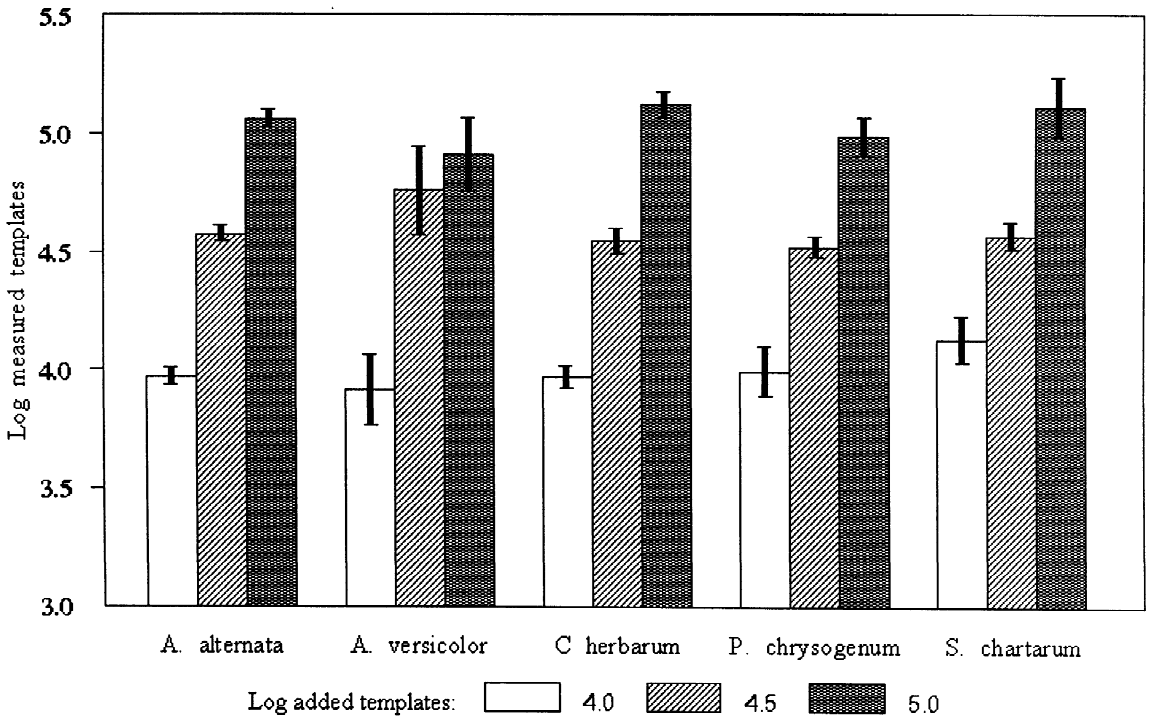


Fig. 4. Validation of QC-PCR for the quantitation of rDNA templates from different organisms based on comparisons of experimentally measured versus expected copy numbers of templates. The histogram shows the $\log_{10}$-transformed quantities and standard errors of rDNA template copies measured by QC PCR analysis in reactions initiated with 1×10^4, 3.3×10^4 and 1×10^5 purified template copies. Each quantity is the mean of at least three replicate measurements.

from 10^4 to 10^5 copies ($P = 0.91 > 0.05$). This indicated that the assay would be suitable for measuring these sequences in unknown samples such as DNA extracts, at least within the validated quantity range.

3.2. Analysis and comparison of rDNA yields from different extraction methods

The five DNA extraction methods selected for this study, were based on different, commonly used plant and fungal cell lysis procedures. A series of preliminary experiments were also performed to compare the rDNA yields from liquid nitrogen-ground conidia using the glass binding DNA purification method described in Section 2.3 with those recovered using a previously described procedure involving incubation of the lysates in SDS buffer, phenol–chloroform extraction and isopropanol precipitation (Lee and Taylor, 1990). Results from these experiments indicated that the glass binding-based purification method recovered from six to thirty-fold greater quantities of templates from equivalent amounts of ground conidia. These results led to the adoption of the glass binding method as a standard component of all extraction methods examined in subsequent experiments. With the exception of freeze–thawing, the lengths of treatment times indicated in Section 2.3 for each of the cell lysis procedures were established from rDNA template yield results obtained in preliminary time course experiments. The bead milling and sonication methods, in particular, showed treatment length optima which, if exceeded, resulted in yield reductions (data not shown).

Table 2 shows the mean template yields, expressed on a per conidium basis, obtained by the different extraction methods from the different test organisms. Analysis of variance of the $\log_{10}$ transformed data showed statistically significant differences in yields among the extraction methods, and significant variation in the relative yields of the methods according to the test organism involved ($P \leq 0.0001$). Overall yields (i.e. the means of the yields from all organisms) were highest using the bead milling method followed by ambient temperature grinding, grinding in liquid nitrogen, sonication and freeze–thawing. Yields using the bead milling method were significantly higher, and using the freeze–thawing method significantly lower, than those of all of the other methods in these comparisons ($P < 0.05$). In comparisons of the data for each of the individual organisms, the relative yield rankings of the different methods and the significance of these rankings varied by organism (Table 2). In these comparisons, however, bead milling was the only method that consistently gave yields that either ranked highest or were not significantly different from the highest ranking method. These results are in accord with other recent studies that have reported bead milling to be relatively effective in

Table 2
PCR template yields[a] from fungal conidia using different cell lysis procedures

Species	BM[b]	AG	N_2	SON	F–T
Aspergillus versicolor	0.80 (50.3) A	0.39 (92.0) A	0.04 (114.6) A	0.08 (121.4) A	0.07 (110.2) A
Penicillium chrysogenum	3.10 (32.3) A	0.03 (100.0) C	0.09 (90.9) B,C	1.92 (70.6) A,B	> 0.01 (92.0) C
Stachybotrys chartarum	11.13 (22.7) A	14.21[c] (83.8) A	9.45 (113.6) A,B	0.85 (130.8) B,C	0.12 (165.2) C
Cladosporium herbarum	23.45 (22.8) A	5.73[d] (165.2) A,B	0.91[d] (77.9) B,C	15.38 (98.2) A,B	0.14[d] (64.9) C
Alternaria alternata	21.09 (19.1) A	22.38 (64.9) A	13.53 (69.6) A	0.25 (94.8) B	0.72 (21.0) A,B
All species	11.91 (29.4)[e] A	8.54 (101.2)[e] B	4.80 (93.3)[e] B	3.70 (103.2)[e] B	0.21 (90.7)[e] C

[a] Yields expressed as PCR template copies recovered per cell. Unless otherwise specified, values shown are means from three replicate extractions per species. Associated coefficients of variation are shown in parentheses. Mean values followed by the same letters in the same rows are not significantly different ($P = 0.05$) based on analysis of $\log_{10}$ transformed data using the Bonferroni test for individual treatment differences.

[b] Cell lysis procedure abbreviations: BM, bead milling; AG, ambient grinding; N_2, liquid nitrogen grinding; SON, sonication; F–T, freeze–thawing.

[c] Mean value from four replicate extractions.

[d] Mean value from five replicate extractions.

[e] Means of the mean yield values and coefficients of variation for individual species listed above.

comparison with other methods at releasing DNA from the spores or conidia of a variety of both bacterial and fungal species (Muller et al., 1998; Kuske et al., 1998).

A second distinguishing feature of the bead milling method was its relatively high precision of template yields from extract to extract. As shown in Table 2, the CV values associated with this method were lower than those corresponding to each of the other methods for each of the test organisms. Comparison of these results using a nonparametric procedure, indicated that this difference was significant ($P < 0.05$). Based on the overall template recovery precision demonstrated by the bead milling method in this study (i.e. the mean of the CV values for all organisms), it is predicted that, at a significance level of 0.05, this method would support the detection of 4-fold differences in rDNA copy numbers in a PCR assay with 95% probability.

Mean yields of templates from organisms with relatively small conidia (i.e. *A. versicolor* and *P. chrysogenum*, Table 1) were generally found to be much lower than those from organisms with relatively large conidia including *S. chartarum*, *C. herbarum* and *A. alternata*. This bias was most evident and statistically significant ($P < 0.05$) in the liquid nitrogen and ambient temperature grinding methods where mean template yields from the smaller conidia were 122 and 67-fold lower, respectively, than those from the larger conidia. Imperfect tolerances between pestles and grinding tubes may explain the lower disruption efficiencies of the smaller cells in these grinding-based methods. Mean template yields from the smaller conidia were also 9-fold lower than those from the larger conidia using the bead milling method. This difference was not found to be significant, however, at the 0.05 α level in the current study.

The results of this investigation suggest that none of the extraction methods examined, including bead milling, are highly efficient in recovering DNA from fungal conidia. While the rDNA copy numbers in the genomes of the test species are not known, reported analyses of several other fungal species have given values ranging from ≈ 50 to > 200 (Clare et al., 1986; Garber et al., 1988; Howlett et al., 1997). Despite this, the quantities of rDNA templates recovered by the bead milling method in this study can be used to predict that this method would allow the detection of < 10 cells of each of the examined species, with 97% confidence. This prediction, however, is based on two assumptions that will require further investigation.

The first assumption is that template recovery levels from all of the conidia of a given target species in a sample are fairly uniform. Other studies being pursued in our laboratory have thus far supported this assumption by demonstrating that quantities of as little as two conidia of *S. chartarum* can be consistently detected against a background of more than 10^5 non-target conidia in PCR assays when using the bead milling extraction method for template recovery (unpublished data).

The second assumption is that the extraction method is effective in separating potential PCR inhibitors from the templates (Wilson, 1997). In this study, a series of failed or clearly inhibited reactions were, in fact, encountered. Interestingly, the majority of these occurrences were found to be associated with the use of distilled water in the reactions that had undergone extended storage in polypropylene tubes. This observation emphasizes previous warnings that have been issued concerning the possible presence of PCR inhibitors in such containers (Chen et al., 1994). A total of three additional instances of PCR inhibition were observed in this study where the results appeared to have been directly caused by the DNA samples. Two of these instances involved extracts obtained from the liquid nitrogen grinding method and one from the ambient grinding method. These methods could potentially facilitate the release of inhibitors present in the walls of the polypropylene tubes used for sample preparation as a result of the grinding action. No instances of sample-related PCR inhibition were observed in association with the bead milling method. Consequently it appears that this method is adequate in removing potential inhibitors associated with the conidia of the test organisms, at least at the cell concentrations used. Further studies are currently being performed to determine the possible effects of external inhibitors that might be encountered in indoor air samples (e.g. dust particles) on quantitative PCR analyses.

4. Conclusions

A DNA extraction method incorporating the lysis of cells by glass bead milling and purification of DNA by selective binding to glass milk particles was found to be superior to several other methods examined in recovering functional PCR templates from the conidia of a variety of fungal species. This method should be capable of supporting highly sensitive assays for the detection of each of these organisms in air samples based on selective PCR amplification of their rDNA sequences.

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