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Journal of Microbiological Methods 50 (2002) 319–323

Journal of Microbiological Methods

www.elsevier.com/locate/jmicmeth

Note

Evaluation of rapid DNA extraction methods for the quantitative detection of fungi using real-time PCR analysis

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Received 10 January 2002; received in revised form 20 February 2002; accepted 20 February 2002

Abstract

Three comparatively rapid methods for the extraction of DNA from fungal conidia and yeast cells in environmental (air, water and dust) samples were evaluated for use in real-time PCR (TaqMan™) analyses. A simple bead milling method was developed to provide sensitive, accurate and precise quantification of target organisms in air and water (tap and surface) samples. However, quantitative analysis of dust samples required further purification of the extracted DNA by a streamlined silica adsorption procedure. Published by Elsevier Science B.V.

Keywords: DNA; Extraction; Fungi; Real time; PCR; Quantitative

Haugland et al. (1999a) described several procedures to prepare PCR-amplifiable DNA from fungal conidia. The most efficient procedure involved disruption of the conidia by bead milling followed by adsorption of the released DNA onto silica particles and multiple washing and centrifugation steps. This method was used for detecting and quantifying the toxigenic mold, *Stachybotrys chartarum*, by real-time PCR analysis using the TaqMan™ PCR product detection system and ABI Prism® model 7700 sequence detection instrument (Haugland et al., 1999b; Vesper et al., 1999; Roe et al., 2001). Briefly, this method involved mixing test and reference fungal conidia suspensions (10 μl each) with 0.3 g of acid-washed glass beads (G-1277; Sigma, St. Louis, MO) and 10, 100 and 300 μl of glass milk suspension, respectively, lysis buffer and binding buffer respectively from an Elu-Quik® DNA purification kit (Schleicher and Schuell, Keene, NH) in sterile, 2-ml conical bottom, screw cap tubes (PGC Scientifics, Gaithersburg, MD). The tubes were shaken in a mini bead beater (Biospec Products, Bartlesville, OK) for 1 min at a maximum rate. In this method, the lysate–glass milk mixtures were incubated with constant tube inversion for 10 min and then transferred to SPIN filter and Catch tube assemblies (Bio101, Vista, CA) for centrifugation at 7500×*g* for 1.5 min. The glass milk particles, with absorbed nucleic acids, were washed twice in the filter cartridges by gentle vortexing with 0.5 ml of Elu-Quik wash buffer and once with 0.5 ml of Elu-Quik salt reduction buffer with centrifugation, as described above, after each step. The nucleic acids were released from the glass particles by vortexing sequentially with two 0.1-ml aliquots of distilled water and collected by centrifug-

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0167-7012/02/$ - see front matter. Published by Elsevier Science B.V.
PII: S0167-7012(02)00037-4

ing the eluates into clean catch tubes. This extraction procedure is fairly labor-intensive and requires approximately 2 h for the routine processing of batches of 12 samples. Our objective was to develop a more rapid method for extracting fungal DNA that would support similar sensitivity and accuracy in real-time PCR analyses of a variety of different environmental samples.

Stock conidia suspensions from 14- to 21-day potato dextrose agar cultures of *S. chartarum* #6417 (University of Alberta Microfungus Collection and Herbarium (UAMH), Alberta, Canada), *Aspergillus fumigatus* #163 (Northern Regional Research Laboratory, Peoria, IL) and *Geotrichum candidum* #7863 (UAMH) were prepared and enumerated by direct microscopic counting in a hemocytometer, as previously described (Roe et al., 2001). Stock cell suspensions from 2- to 3-day yeast maltose agar cultures of *Candida albicans* #18804 (American Type Culture Collection, Rockville, MD) were similarly enumerated.

In Method 1, 200 μl of PrepMan™ Ultra reagent (Applied Biosystems, Foster City, CA) was added to a 2-ml conical bottom, screw cap tube along with cells or conidia of the target species. The PrepMan reagent was developed by its manufacturer to facilitate the microbial cell lysis and to remove PCR inhibitors from food samples but, to our knowledge, has not been tested on environmental samples. The tube was then vortexed for 10 s at a maximum speed. Method 2 was set up similar to Method 1 but 0.3 g of glass beads was also added to the 2-ml tube. The tube was shaken in a mini bead beater for 1 min at a maximum rate. In some tests of Method 2, 200 μl of sterile, deionized water was substituted for the PrepMan reagent. The extract tube from Method 1 or 2 was then incubated for 10 min at 100 °C, then for 2 min at room temperature, then centrifuged for 3 min at 16,000×*g*. The supernatant above the beads was then recovered for TaqMan analysis.

In Method 3, 100 μl of lysis buffer and 300 μl of binding buffer from the Elu-Quik® kit were added to the bead beating tube plus the fungal cells or conidia and 0.3 g of beads. The tubes were shaken in the mini bead beater, as described above for Method 2. This product was incubated for 10 min at 70 °C, then centrifuged for 1 min at 8000×*g*. The recovered solution (above the beads) was mixed with 160 μl of cold ethanol and transferred to a DNeasy™ glass filter column (Qiagen, Valencia, CA, USA) on a QIAvac 24 manifold (Qiagen). The column was washed twice with 0.5 ml of the Qiagen AW1 buffer and once with 0.5 ml of the AW2 buffer, then centrifuged for 2 min at 16,000×*g*, transferred to a clean catch tube and the DNA recovered by adding 0.1 ml Qiagen AE buffer, centrifuging the column for 1 min at 8000×*g* and then repeating this steps with another 0.1 ml of AE buffer to give a final recovered volume of 0.2 ml.

TaqMan assays were prepared in 0.5 ml thin-walled optical grade PCR tubes (Applied Biosystems) by the addition of the following components: 12.5 μl of TaqMan Universal Master Mix, a 2× concentrated, proprietary mixture of AmpliTaq Gold™ DNA polymerase, AmpErase® UNG, dNTPs with UTP, passive reference dye and optimized buffer components (Applied Biosystems); 5 μl of a mixture of forward and reverse primers (5 μM each) and 400 nM TaqMan probe; 2.5 μl of 2 mg/ml bovine serum albumin (fraction V, Sigma) and 5 μl of DNA template.

Primers and probes for the detection of *S. chartarum* and *G. candidum* sequences have been described (Haugland et al., 1999b). The primers and probe for *A. fumigatus* were: 5′-GCCCGCCGTTTCGAC (AfumiF1, forward primer); 5′-GTTGTTGAAAGTTTTAACTGATTC (AfumiR1, reverse primer) and 5′-CCCGCCGAAGACCCCAACATG (AfumiP1, probe). These sequences are homologous to the rDNA internal transcribed spacer 1 (ITS1) region of *A. fumigatus* and thus far have been found to only detect this species and *Neosartorya fischeri* in analyses of a wide range of *Aspergillus* and related species (unpublished data).

The primers and probe for *C. albicans* were: 5′-CTTGGTATTTTGCATGTTGCTCTC (CalbF1, forward primer), 5′-GTCAGAGGCTATAACACACAGCAG (CalbR1, reverse primer) and 5′-TTTACCGGGCCAGCATCGGTTT (CalbP1, probe). These sequences are homologous to the D1 region of the *C. albicans* large subunit ribosomal RNA gene (Kurtzman and Robnett, 1997) and thus far have been found to only detect this species in analyses of a wide range of *Candida* and related species (Brinkman et al., 2001).

Relative quantities of target sequences in cell or conidia extracts and calibrator samples were deter-

Table 1
Recoveries of rDNA, target sequences by different extraction methods[a]

Species	Method 1	Method 2	Method 3	Published method
A. fumigatus	0.02 (0.01–0.03)	18.10 (12.32–26.6)	10.74 (5.96–19.36)	11.64 (7.68–17.64)
C. albicans	0.05 (0.03–0.08)	10.87 (7.48–15.80)	2.85 (1.65–4.93)	6.16 (3.99–9.49)
G. candidum	0.10 (0.07–0.17)	24.13 (16.19–35.95)	10.82 (6.33–18.48)	25.75 (16.99–39.02)
S. chartarum	1.13 (0.64–1.98)	86.02 (51.80–142.85)	23.85 (13.61–41.81)	29.84 (18.00–49.47)

[a] Mean estimated copy numbers recovered per cell or conidium from three replicate extracts (two replicate TaqMan analyses per extract) with standard deviation ranges shown in parentheses. Extracts were from 20,000 conidia of *A. fumigatus*, 2000 cells of *C. albicans*, 2,000,000 conidia of *G. candidum* and 3000 conidia of *S. chartarum*. Procedures for the extraction methods are described in the text.

mined from their respective cycle threshold (C_T) values in TaqMan assays by means of the comparative C_T method (Anonymous, 1997). This analysis uses the formula $E^{-\Delta C_T}$, where E is equal to the amplification efficiency of the assay and ΔC_T is equal to the C_T value of the cell or conidia extract minus the C_T value of the calibrator sample. The amplification efficiency (E) for each primer set was determined in preliminary experiments from the slopes of plots of C_T values versus serially diluted DNA extracts of each fungus (data not shown).

Table 1 shows the estimated number of target sequence copies recovered with the three new DNA extraction procedures and our previously published bead milling procedure (Haugland et al., 1999a) as determined by the comparative C_T method. Calibrator samples in these experiments contained known copy numbers of purified rDNA amplicons derived from conventional PCR reactions that spanned the respective target DNA regions for the different TaqMan assays. *C. albicans* amplicons were generated using the primers NL11 and NL21, whereas amplicons used for the analyses of other organisms were generated from the primers NS91 and IT60 (Haugland et al., 2001). Copy numbers of these amplicons in the calibrator samples were determined by subjecting aliquots of the amplicon stock solutions to gel electrophoresis and fluorimaging analysis with mass standards (Haugland et al., 1999a).

Significantly lower copy yields ($P<0.0001$) were obtained using Method 1 compared with all other methods for all organisms, so this method was eliminated from further consideration. Method 3 gave results comparable to our previously described procedure (Table 1). Method 2 was slightly better than the other methods in recovering DNA. For Method 2, the use of water in the extraction (instead of the PrepMan reagent) was just as effective (Table 2). Because both Methods 2 and 3 were relatively rapid and comparable in target sequence yields to our previous method, their use with environmental samples (air, water and dust) was further tested.

Outdoor air particulates were collected from three 2000-l samples using a liquid impingement type air sampling device and concentrated by centrifugation, as previously described (Haugland et al., 1999b). Water particulates were concentrated by centrifugation in the same manner from three 100-ml tap water samples with turbidity readings of 2.4 nephelometric turbidity units (NTU) and from three 50-ml surface water samples with turbidity readings of 4.5 to 12.9 NTU. Dust was obtained from three heating, ventilation and air conditioning (HVAC) sources. These air, water and dust samples were spiked with approximately 100 and 500 conidia or cells each of *A. fumigatus*, *S. chartarum* and *C. albicans*, and subjected to DNA extraction by Methods 2 and 3.

The accuracy and precision of the TaqMan assay-based quantitative measurements of the target organisms, *A. fumigatus* (Af), *S. chartarum* (Sc) and *C. albicans* (Ca), in the different samples are summarized as the ratios of the numbers of measured cells or

Table 2
Comparison using Method 2 with water versus PrepMan™ reagent for the extraction efficiency of fungi spiked in surface water[a]

	C_T with water	C_T with PrepMan
A. fumigatus	26.2±0.5	27.8±1.2
C. albicans	27.8±0.2	27.5±0.1
S. chartarum	25.2±0.1	28.2±0.1

[a] Mean estimated C_T value plus standard deviation of recovered target sequences from two replicate TaqMan analyses of extracts of 50 ml, 12.9 NTU surface water samples amended with 500 cells or conidia of each organism and concentrated as described in the text.

conidia in the samples to the numbers added to the samples (Fig. 1). Measured numbers in the environmental samples were determined from comparative C_T analyses of DNA extracts of these samples and of calibrator samples which, in these experiments, consisted of known cell or conidia quantities of the same organisms in distilled water (Haugland et al., 1999b). As in previous applications of this analysis method, *G. candidum* conidia were added to both the environmental and calibrator samples in relatively high numbers (2×10^6 conidia per sample) prior to the extraction, to act as internal references to normalize the assay results of the target organisms for any sample-to-sample variations in the overall DNA extraction efficiencies and also to detect potential PCR inhibitors in the extracts, as previously explained (Roe et al., 2001). Low quantities of naturally occurring target cells or conidia were detected in some of the same surface water and dust samples that received no amendments. These quantities were also determined by comparative C_T analyses and subtracted from the measured quantities in the amended samples prior to calculating the ratios.

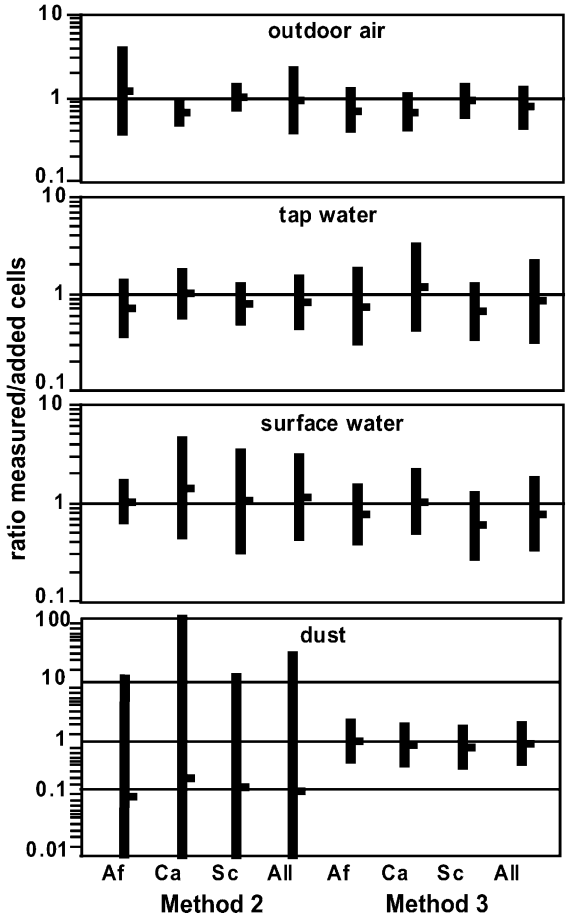

Fig. 1. Ratios of the measured to added *C. albicans* (Ca) cells and *A. fumigatus* (Af) and *S. chartarum* (Sc) conidia in various environmental matrices. Mean quantities were determined for the six analyses of each target organism (three samples×two added cell or conidia quantities) and for the 18 analyses of all three organisms (All), and are indicated by the horizontal lines in the panels for each sample type. Associated 95% occurrence ranges of these ratios (the range of values within which 95% of the individual analysis results would be predicted to occur, based on the standard deviations of each data set) are indicated by the vertical lines.

Ratios obtained in the analyses of the air and water samples were compared using extraction Methods 2 and 3. Overall mean ratios for all target organisms in these samples were 0.98 and 0.80 for Methods 2 and 3, respectively. The 95% occurrence ranges of the ratios were 0.44–2.20 and 0.39–1.66 for the two methods, respectively. However, the results obtained for dust samples using Methods 2 and 3 were very different.

Dust samples extracted with Method 3 showed ratios with a mean of 0.88 and 95% occurrence range of 0.44–1.77 (similar to the air or water samples described above). In contrast, dust samples prepared by Method 2 often gave either negative results or highly variable C_T values. This resulted in ratios with a considerably lower mean of 0.09 and wider 95% occurrence range of 0.00028–27.49. These differing results between Methods 2 and 3 were explained by the internal reference (*G. candidum*) assay data.

No major increases were observed in the *G. candidum* C_T values from the air and water samples, extracted by either Method 2 or 3, compared with those of the corresponding calibrator samples (data not shown). This indicated that overall DNA recoveries from these samples and the calibrator samples were comparable and that inhibitors were either not present in these low particulate background samples or were eliminated by both methods. Similarly, none of the dust samples extracted by Method 3 showed increases in the *G. candidum* C_T values. In contrast, the *G. candidum* assay C_T values from all three dust samples extracted by Method 2 were either negative or substantially higher than those of the calibrator samples. Analyses of dilutions of these extracts, as

previously described (Roe et al., 2001), showed that PCR inhibitors in these samples were not fully eliminated.

Thus, while Methods 2 and 3 were equally useful for fungal DNA extractions from air or water samples, the additional purification steps in Method 3 were required for the analyses of dust samples. Our previously reported DNA extraction procedure for fungal conidia (Haugland et al., 1999a) required 2 h or more to prepare 12 samples. For samples with low concentrations of PCR inhibitors, Method 2 cuts the preparation time to about 45 min for 12 samples with a potential increase in sensitivity and accurate quantitative results in subsequent TaqMan analyses. Method 3, with a processing time of about 90 min for 12 samples, provides smaller but still significant savings in time and labor, similar accuracy and only a small potential reduction in sensitivity. As such, this method offers a practical compromise in choosing among speed, sensitivity and general applicability in the analysis of different types of environmental samples by real-time PCR.

Acknowledgements

This research was supported in part by the appointment of N.B. to the Postgraduate Research Participation Program administered by the Oak Ridge Institute for Science and Education through an interagency agreement between the U.S. DOE and the U.S. EPA.

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