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Evaluation of a Rapid, Quantitative Real-Time PCR Method for Enumeration of Pathogenic *Candida* Cells in Water

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Quantitative PCR (QPCR) technology, incorporating fluorigenic 5′ nuclease (TaqMan) chemistry, was utilized for the specific detection and quantification of six pathogenic species of *Candida* (*C. albicans*, *C. tropicalis*, *C. krusei*, *C. parapsilosis*, *C. glabrata* and *C. lusitaniae*) in water. Known numbers of target cells were added to distilled and tap water samples, filtered, and disrupted directly on the membranes for recovery of DNA for QPCR analysis. The assay's sensitivities were between one and three cells per filter. The accuracy of the cell estimates was between 50 and 200% of their true value (95% confidence level). In similar tests with surface water samples, the presence of PCR inhibitory compounds necessitated further purification and/or dilution of the DNA extracts, with resultant reductions in sensitivity but generally not in quantitative accuracy. Analyses of a series of freshwater samples collected from a recreational beach showed positive correlations between the QPCR results and colony counts of the corresponding target species. Positive correlations were also seen between the cell quantities of the target *Candida* species detected in these analyses and colony counts of *Enterococcus* organisms. With a combined sample processing and analysis time of less than 4 h, this method shows great promise as a tool for rapidly assessing potential exposures to waterborne pathogenic *Candida* species from drinking and recreational waters and may have applications in the detection of fecal pollution.

Yeasts are a significant component of the microbiota of most natural aquatic ecosystems (17, 33) and can also occur in drinking water distribution systems as a result of their ability to survive treatment practices and become incorporated into biofilms (6, 12, 22, 30, 31). The majority of these organisms have no known human health effect. However, a small number of species, primarily within the anamorphic genus *Candida*, are important opportunistic pathogens (23).

The importance of pathogenic *Candida* as agents of nosocomial infections has led to the development of a number of modern molecular diagnostic methods to facilitate their detection and identification in clinical samples. Methods based on the PCR and DNA hybridization probes have received particular attention (9, 25, 26, 32, 39). The more recent advent of fluorescent probe-based PCR technology (21) has led to the development of homogeneous methods for detecting these organisms that require relatively short periods of time to perform (16, 28).

Quantitative PCR (QPCR) has been demonstrated to be useful for quantitative analysis of microorganisms in environmental samples (29, 34, 35, 36), but, to our knowledge, this approach has not been used in the analysis of yeasts in water. Analyses for pathogenic yeasts in drinking or recreational water systems have the potential to expedite the identification of possible health hazards resulting either directly from the presence of these organisms or, as their presence might indicate, indirectly from other waterborne pathogens.

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The first objective of this study was to develop QPCR technology, incorporating fluorigenic 5′ nuclease (TaqMan) chemistry, for specifically detecting and quantifying six common pathogenic species of *Candida*, namely, *C. albicans*, *C. tropicalis*, *C. krusei*, *C. parapsilosis*, *C. glabrata*, and *C. lusitaniae*. The second objective was to evaluate a simple and rapid method, using QPCR, for the detection and enumeration of these organisms in different types of water samples. Finally, the method was compared with conventional plating and culturing methods in the analysis of a series of freshwater samples collected from a recreational beach on Lake Michigan.

MATERIALS AND METHODS

Yeast cultures. The strains used in this study are listed in Table 1. For preparation of cell stocks, *Geotrichum candidum* cultures were grown for several weeks on potato dextrose agar (Becton and Dickinson, Fairfax, Va.), and the other cultures were grown on yeast mannitol agar (Becton and Dickinson) for 24 to 48 h at room temperature. Cells were harvested by using a moistened, sterile cotton swab and resuspended in sterile water containing 0.05% Tween 80. Suspended cell stock concentrations were determined by counting in a hemocytometer chamber at 400× magnification as previously described (29), and 50- to 100-μl aliquots were stored at −80°C. Aliquots of *G. candidum* stocks, containing 2×10^6 cells, and aliquots of different *Candida* and other yeast cell stocks, containing between 10^4 and 10^5 cells, were added as external references and target organisms, respectively, to calibrator samples for QPCR analysis, as previously described (19, 29), or were used as sources of known cell quantities for various experiments described below.

Cell suspension and collection filter extractions. Yeast cell suspensions used as calibrator samples or for determining assay specificity, amplification efficiency, and cell detection limits were extracted by a rapid bead-milling method (20). Ten-microliter aliquots of both target yeast and *G. candidum* reference cell stocks were combined with 200 μl of AE buffer (Qiagen, Valencia, Calif.) in a 2.0-ml conical-bottom, screw-cap tube (PGC Scientifics, Gaithersburg, Md.) containing 0.3 g of acid-washed glass beads (Sigma, St. Louis, Mo.). The tubes were shaken in a mini bead-beater (Biospec Products, Bartlesville, Okla.) for 1 min at

TABLE 1. Fungal cultures, sources, and GenBank accession numbers of organisms used in this research

Species	Source and strain no.[a]	GenBank sequence accession no.
C. albicans	ATCC 18804	U45776
C. albicans	ATCC 11006	
C. albicans	ATCC 14053	
C. albicans	ATCC 24433	
C. albicans	ATCC 36232	
C. albicans	ATCC 60193	
C. albicans	ATCC 66027	
C. dubliniensis	NRRL Y-17841	U57685
C. glabrata	ATCC 2001	U44808
C. glabrata	ATCC 66032	
C. glabrata	NRRL Y-17815	
C. guilliermondii[b]	NRRL Y-2075	U45709
C. guilliermondii	ATCC 6260	
C. haemulonii	NRRL Y-6693	U44812
C. haemulonii type II	NRRL Y-17801	U44819
C. insectamans	ATCC 22874	U45753
C. krusei[c]	NRRL Y-5396	U76347
C. krusei	ATCC 6258	
C. krusei	ATCC 14243	
C. lipolytica[d]	NRRL YB-423	U40080
C. lusitaniae[e]	NRRL Y-11827	U44817
C. lusitaniae	ATCC 42720	
C. lusitaniae	ATCC 66035	
C. lusitaniae	NRRL Y-5393	
C. lusitaniae	NRRL Y-11826	
C. lyxosophila	NRRL Y-17539	U76204
C. maltosa	NRRL Y-17677	U45745
C. maltosa	UGA R-42	
C. parapsilosis	ATCC 22019	U45754
C. parapsilosis	NRRL Y-7363	
C. parapsilosis	NRRL Y-543	
C. sake	ATCC 14478	U45728
C. sojae	NRRL Y-17909	U71070
C. tropicalis	ATCC 750	U45749
C. tropicalis	UGA 52-71	
C. tropicalis	ATCC 13803	
C. tropicalis	ATCC 66029	
C. viswanathii	ATCC 22981	U45752
C. zeylanoides	NRRL Y-1774	U45832
Cryptococcus neoformans	ATCC 14116	AF189845
Geotrichum candidum	UAMH 7863	AF157596
Pichia angusta	ATCC 34438	U75524
Lodderomyces elongisporus	ATCC 11503	U45763
Rhodotorula mucilaginosa	ATCC 9449	AF189960
Trichosporon cutaneum	ATCC 28592	AF075483

[a] ATCC, American Type Culture Collection; NRRL, Northern Regional Research Laboratory, U.S. Department of Agriculture; UGA, University of Georgia; UAMH, University of Alberta Microfungus Collection and Herbarium.
[b] *Pichia guilliermondii*, as renamed (24).
[c] *Issatchenkia orientalis*, as renamed (24).
[d] *Yarrowia lipolytica*, as renamed (24).
[e] *Clavispora lusitaniae*, as renamed (24).

the maximum rate and then centrifuged in an Eppendorf microcentrifuge at 14,000 rpm for 3 min. The genomic DNA in the supernatant above the beads was transferred to a sterile microfuge tube and stored at −80°C. In some cases, the extracts were further purified by use of a Qiagen purification kit procedure. This procedure was performed by adding 300 μl of binding buffer from an Elu-Quik DNA purification kit (Schleicher and Schuell, Keene, N.H.) to 100 μl of the supernatant indicated above and purifying on a DNeasy glass filter column (Qiagen), as previously described (20).

Collection filter extractions (CFE) were performed with polycarbonate filters (Osmonics Inc., Minnetonka, Minn.) used to recover cells from water samples. After filtration of the water samples on a manifold device, the filters were placed in 2.0-ml conical-bottom, screw-cap tubes containing 0.3 g of acid-washed glass beads, 10 μl of *G. candidum* reference cell stock, and 200 μl of AE buffer; they were then disrupted by bead milling, and DNA was recovered, as described above. These extracts were also further purified, in some instances, by use of a Qiagen purification kit procedure (CFE+Q), as described above.

Design of TaqMan primer and probe sets. The QPCR assays targeted the variable D1/D2 domains of the nuclear large subunit (LSU) ribosomal gene. Sequences from virtually all known ascomycetous yeast species have been determined for this region (24), which facilitated the design and testing of the assays for species specificity. Table 1 lists the GenBank accession numbers for the target species and other organisms that were experimentally examined. Phylogenetic analysis, based on the LSU DNA sequences, has placed the six target species in four different clades (24). Therefore, four alignments were made that included sequences of the target species and those of relatives in their respective clades by using the MegAlign program of the Lasergene Biocomputing software package (DNAStar, Inc., Madison, Wis.). Manual searches of the alignments were conducted to determine species-specific candidate primer sequences. The candidate primer sequences for each of the target species were analyzed in the Oligo 6 primer analysis software program (Molecular Biology Insights, Inc., Cascade, Colo.) for primer stability (e.g., optimal melting temperature, potential primer-dimer formation, and hairpin structures) and predictions of false priming of the corresponding sequences of all nontarget species in their respective clades (18, 19). Final selection of primer and probe sequence lengths and determinations of their compatibility with the fluorigenic 5′ nuclease assay were performed by using the ABI Primer Express program (Applied Biosystems, Foster City, Calif.). The primer and probe sequences in Table 2 were custom synthesized at the Applied Biosystems oligonucleotide factory. The probes contained the reporter dye 6-FAM linked to the 5′-terminal nucleotide and the quencher dye TAMRA conjugated to the 3′ end. The primers and probe used in the *G. candidum* reference assay have been previously described (19).

QPCR reactions. Reactions were performed in 0.5-ml thin-walled, optical-grade PCR tubes (Applied Biosystems) by 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, deoxyribonucleoside triphosphates with UTP, passive reference dye, and optimized buffer components; 5 μl of a mixture of forward and reverse primers (5 μM each) and 400 nM TaqMan probe; and 2.5 μl of a 2-mg/ml concentration of bovine serum albumin (fraction V, Sigma) and 5 μl of DNA template. The reactions were monitored in an Applied Biosystems Prism model 7700 sequence detection instrument. Thermal cycling conditions consisted of 2 min at 50°C and 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Determinations of cycle threshold (C_T) were performed automatically by the instrument.

Quantities of target cells in each test sample were determined by using the $\Delta\Delta C_T$ comparative cycle threshold method, as previously described (User Bulletin no. 2 [1997], ABI Prism 7700 Sequence Detection System, Applied Biosys-

TABLE 2. The QPCR primer and probe sequences developed for the identification and quantification of pathogenic *Candida* species in water

Target species (assay abbreviation)	Primer/probe name[a]	Primer/probe sequence
C. albicans (Calb)	Calb F1	5′-CTTGGTATTTTGCATGTTGCTCTC-3′
	Calb R1	5′-GTCAGAGGCTATAACACACAGCAG-3′
	Calb P1	5′-TTTACCGGGCCAGCATCGGTTT-3′
C. glabrata (Cglab)	Cglab F1	5′-GCGCCCCTTGCCTCTC-3′
	Cglab R1	5′-CCCAGGGCTATAACACTCTACACC-3′
	Cglab P1	5′-TGGGCTTGGGACTCTCGCAGC-3′
C. parapsilosis (Cpar)	Cpar F1	5′-GATCAGACTTGGTATTTTGTATGTTACTCTC-3′
	Cpar R1	5′-CAGAGCCACATTTCTTTGCAC-3′
	Cpar P1	5′-CCTCTACAGTTTACCGGGCCAGCATCA-3′
C. tropicalis (Ctrop)	Ctrop F1	5′-GCGGTAGGAGAATTGCGTT-3′
	Ctrop R2	5′-TCATTATGCCAACATCCTAGGTTTA-3′
	Ctrop P2	5′-CGCAGTCCTCAGTCTAGGCTGGCAG-3′
C. lusitaniae (Clus)	Clus F2	5′-GGGAATTGTAATTTGAAGGTTTCGT-3′
	Clus R2	5′-GTCGGCGTGCGCCATA-3′
	Clus P2	5′-TCTGAGTCGGCCGCGCCC-3′
C. krusei (Ckru)	Ckru F1	5′-CTCAGATTTGAAATCGTGCTTTG-3′
	Ckru R1	5′-GGGGCTCTCACCCTCCTG-3′
	Ckru P1	5′-CACGAGTTGTAGATTGCAGGTTGGAGTCTG-3′

[a] F, forward primer; R, reverse primer; P, probe.

tems) and then modified (19, 29). Briefly, this method determines the relative quantity of target DNA sequences extracted from an unknown test sample compared to the quantity of target sequences extracted from a known quantity of target organisms in a calibrator sample. This is done after normalizing for the relative recoveries of total DNA in the extraction process from the two samples by comparing the recovered quantities of another sequence from an external reference organism added in equal cell numbers to both. For each test sample and corresponding calibrator sample reaction, a ΔC_T value is obtained by subtracting the C_T value of the reference sequence assay ($C_{T\text{ref}}$) from the C_T value of the target sequence assay ($C_{T\text{target}}$). A $\Delta\Delta C_T$ value is obtained by subtracting the ΔC_T value of the calibrator sample from the ΔC_T value of the test sample. The ratio of target sequences in the test and calibrator samples is described by $E^{-\Delta\Delta C_T}$, where E is the amplification efficiency of the target assay. These ratios are multiplied by the known number of target organism cells in the calibrator samples to obtain estimates of the numbers of target cells in each test sample.

Specificity of *Candida* QPCR assays. The specificity of each *Candida* assay was verified experimentally by using the array of species listed in Table 1. DNA extracts were prepared as described above from 10 μl of the suspended cell stocks of one representative strain (the type strain in all cases where available) from each species mixed with *G. candidum* reference cells. These extracts were analyzed in duplicate reactions on the model 7700 Sequence Detector for each of the six target species assays (the appropriate primers and probe are as shown in Table 2). A C_T value of 40 (maximum number of PCR cycles run) indicated that DNA from the test strain was not detected.

Amplification efficiencies and extrapolated sensitivities of *Candida* QPCR assays. DNA extracts were prepared, as described above, from suspended cell stocks of each of the target species strains listed in Table 1. Tenfold serial dilutions of these DNA extracts were analyzed by QPCR by using the appropriate primer and probe sets. Each of the undiluted extracts was also analyzed by using the *G. candidum* reference assay. ΔC_T values were calculated by subtracting the reference assay result for each extract from the target assay results for each dilution from that extract. For the purpose of estimating minimum cell detection limits, the mean reference C_T result for all strains was added back to each of the individual ΔC_T values to give normalized target assay C_T values. These values were plotted against the log transformed cell equivalents in the samples (i.e., the target cell numbers in the original extracts divided by the extract dilution factor) to calculate amplification efficiencies and extrapolated cell detection limits.

Ideally the amplification efficiency (E) of a PCR assay is equal to 2; that is, there is a doubling of the number of copies during each cycle (User Bulletin no. 2 [1997], ABI Prism 7700 Sequence Detection System). However, a single cycle may result in less than a doubling, and the latter of the two equations below can be used to estimate E. The relationship of the difference in cycle threshold between target and reference cells (ΔC_T) to the number of target cells or cell equivalents (N) in an extracted sample is related to amplification efficiency by the equation $N = M \cdot E^{-\Delta C_T}$ (where M is a constant of proportionality), or by the equation $\Delta C_T = C_{T\text{ target}} - C_{T\text{ref}} = a + b \cdot \log_{10}(N)$, where $a = \log_{10}(M)/\log_{10}(E)$ and $b = -1/\log_{10}(E)$. This latter equation also can be used to ascertain whether the amplification efficiency is the same for two different organisms, since in that case their respective slopes will be equal, i.e., their regressions will be parallel.

The sensitivity or minimum detection limit of this method is dependent on the threshold being attained within 40 cycles, i.e., $C_T < 40$. Thus, extrapolation of the regression line (by the latter equation above) to a C_T value of 40 provides an estimate of the minimum cell detection limit for each strain. The value of the detection limit, therefore, depends on the intercept and slope of this regression, as well as the extraction efficiency and quality of the DNA in the extracts, which are reflected in the reference cycle threshold, $C_{T\text{ref}}$. If assays among different strains of the same species are parallel, then, for any given level of extraction efficiency and DNA quality, the detection limits of the various strains will differ only if their intercepts differ.

Experimental analysis of assay sensitivity or minimum detection limit. To experimentally assess the sensitivity or minimum detectable cell number, solid-phase cytometry with a Scan RDI instrument (Chemunex, Maisons Alfort, France) was used. Solid-phase cytometry involves the direct detection and enumeration of fluorescently labeled cells that have been collected on membrane filters. The Scan RDI instrument automates this process and allows for rapid visual confirmation by microscopy of the fluorescently detected cells.

Cultures of *C. albicans* were grown for 24 to 48 h in yeast-mannitol broth at 37°C in a shaking incubator. The cultures were then washed twice with 1 ml of filter-sterilized Isoton (Coulter, Miami, Fla.) at a pH of 8.0 and collected by centrifugation at 14,000 rpm. The washed cells were suspended in Isoton, and aliquots were counted in a hemocytometer chamber. Dilutions with different cell concentrations were made from the suspensions, and 1-ml volumes were stained with 5 μl of the general nucleic acid stain Syto 16 (Molecular Probes, Eugene, Oreg.) for 10 min and then filtered through 25-mm-diameter, 0.4-μm-pore-size, polyethylene terephthalate membranes filters (ChemFilter, Chemunex). The cells were enumerated by using a Scan RDI in accordance with the manufacturer's instructions. Each cell identified by the instrument was manually validated by microscopy at 400× magnification with a Nikon Eclipse E600 epifluorescence microscope (Fryer, Inc., Huntley, Ill.) by using a 450- to 490-nm excitation filter and a >520-nm emission filter. The cells on the collection filters were then extracted by using the CFE method and quantified by QPCR and by the $\Delta\Delta C_T$ comparative C_T calculation as described above.

Analysis of tap and surface water samples. Tap, pond, and river water samples were collected locally in Cincinnati, Ohio. Water samples were collected by following quality control guidelines described in sections 9600 A and B of *Standard Methods for the Examination of Water and Wastewater* (15). Turbidity readings for the water samples were made by using a model 2100N tubidimeter (Hach, Ames, Iowa) and were expressed in nephelometric turbidity units (NTU). Different dilutions of *C. albicans* stock cell suspensions were enumerated by solid-phase cytometry, and from these enumerations, known cell quantities in the diluted cell suspensions were spiked into the water samples. The spiked water samples (1,000 ml of tap, 100 ml of pond, and 20 ml of river water) were filtered through 47-mm-diameter, 0.4-μm-pore-size polycarbonate filters (Osmonics Inc.). Both the CFE and CFE+Q methods, described above, were used to recover DNA.

Inhibition of QPCR reactions was evaluated by preliminary analyses of parallel dilutions of each test sample and calibrator extract with the *G. candidum* reference assay. Determinations of appropriate dilutions required for relief of inhibition were made by finding the dilution where the test sample and calibrator $C_{T\text{ref}}$ values coincided (29). Test sample and calibrator extracts of the appropriate dilution were then analyzed with the target and reference assays, and target cells were enumerated by using the $\Delta\Delta C_T$ comparative C_T calculation.

Collection of Lake Michigan beach water samples. Grab samples of Lake Michigan beach water were collected weekly at Mount Baldy beach in Indiana from August 13, 2001 until September 24, 2001. Samples were collected by following the general quality control guidelines indicated above and those in section 3 of *Improved Enumeration Methods for the Recreational Water Quality Indicators: Enterococci and Escherichia coli* (38).

Enumeration of viable *Enterococcus* organisms in Lake Michigan beach water samples. *Enterococcus* populations were determined in each of the Lake Michigan water samples by using EPA Method 1600 (37). Water samples (3 to 100 ml) were filtered through 47-mm-diameter, 0.45-μm-pore-size nitrocellulose filters. The filters were placed on mEI agar (Hach) and incubated at 41°C for 24 h. Enumeration of the enterococci was performed by counting any colonies with a blue halo, in accordance with the method instructions.

Enumeration of organisms of pathogenic *Candida* species in Lake Michigan beach water samples. Enumeration of CFU of target species was performed by using a modification of the ASTM standard method for enumeration of *Candida* species in water (1). This method consisted of filtering water samples (100 to 300 ml) through 47-mm-diameter, 0.45-μm-pore-size nitrocellulose filters (Pall, East Hills, N.Y.), placing the filters on the surface of BiGGY agar (Becton and Dickinson) with 5 mg of chloramphenicol/ml, and incubating at 37°C for 48 h. We replaced the mCA medium specified in the standard method because earlier studies in our laboratory had demonstrated that at 37°C, BiGGY agar with chloramphenicol supports the growth of all of the target species in this investigation, while suppressing growth of bacteria.

To quantify the colony-forming population of each species of *Candida* in the sample, up to 50 *Candida* colonies were randomly picked from each of the plated samples. The isolates were streaked for individual colonies on BiGGY agar and incubated at 37°C for 48 h. Isolated colonies from these plates were inoculated into yeast-mannitol broth and incubated with shaking for 48 h at 37°C. Before the cells in the broth cultures were harvested, optical density readings of each culture were taken at 560 nm with a Spectronic Genesys 5 spectrometer (Spectronic Instruments, Rochester, N.Y.). Based on these readings, different volumes of the cultures were diluted to 1 ml with distilled water so that approximately the same quantities of cells were harvested for each isolate. Culture medium was removed from the diluted cell suspensions after centrifuging for 3 min at 14,000 rpm, and the cell pellets were resuspended in 1.0 ml of sterile, distilled water. Aliquots of the suspensions were extracted as described above without purification and analyzed by the panel of QPCR assays for the six different target species. These analyses allowed the cultured isolates of each of the target species to be identified on the basis of their responses (i.e., C_T values) in the different assays. From these analyses, the percentage of each target species making up the sampled population was determined and these percentages were then multiplied by the

TABLE 3. Assay, amplification efficiency, and mean extrapolated cell detection limits of *Candida* species tested by using the QPCR method[a]

Yeast species	Assay	Amplification efficiency[b]	Mean cell detection limit ± SD[b]
C. albicans	Calb	1.95	1.07 ± 0.74
C. glabrata	Cglab	2.00	0.47 ± 0.11
C. krusei	Ckru	1.89	0.55 ± 0.091
C. lusitaniae	Clus	1.90	0.73 ± 0.46
C. parapsilosis	Cpar	1.92	0.25 ± 0.02
C. tropicalis	Ctrop	1.91	0.083 ± 0.04

[a] Cell detection for all other species tested, as listed in Table 1, were negative. DNA extracts from at least 10^4 cells of each of the nontarget species were analyzed.

[b] Amplification efficiencies and extrapolated cell detection limits were determined as described in the text and illustrated in Fig. 1.

total number of CFU counted from the respective water sample platings to obtain estimates of the number of CFU attributable to each species.

For direct QPCR analysis of target *Candida* species in the Lake Michigan water samples, 100- to 300-ml volumes were filtered through polycarbonate filters, and the filters were extracted by the CFE method. Inhibition of QPCR reactions was determined as described above. Test sample extracts requiring more than a tenfold dilution were further purified by using the CFE+Q method. The variously processed and diluted test sample extracts and correspondingly processed and diluted calibrator extracts were analyzed with the target and reference QPCR assays, and target cell quantities in the test samples were determined by the $\Delta\Delta C_T$ comparative C_T calculation.

Species verification of PCR amplicons from Lake Michigan water samples by sequence analysis. DNA extracts from the water samples collected during weeks 1 and 4 of the study, which gave the most diverse ranges of positive QPCR results for the different target *Candida* species, were amplified with a conventional PCR kit (Expand High Fidelity, Boehringer Manheim, Indianapolis, Ind.) by using the same species-specific primer sets without probes and otherwise identical reaction conditions. The double-stranded products were visualized by agarose gel electrophoresis and SYBR Green I (BioWhitaker Molecular Applications, Rockland, Maine) staining and subjected to purification and direct nucleotide sequencing analysis with the same primers in an Applied Biosystems model 373A DNA sequencer, as previously described (18).

RESULTS

Specificity, amplification efficiency, and extrapolated minimum detection limits of QPCR assays. None of the different *Candida* QPCR assays were positive for any of the organisms listed in Table 1 except for strains of the intended target species listed in Table 2. These results were consistent with the predictions of the Oligo 6 primer analysis program in all instances (results not shown). Analyses in the Oligo 6 program of these primer and probe sets with the rDNA sequences of other species, unavailable for experimental analysis in this study but shown by phylogenetic analyses to be related to the target species (24), indicated that these species would also fail to be detected.

Table 3 shows the amplification efficiencies and average minimum cell detection limits for each assay, as determined from regressions of log transformed cell equivalents on the normalized C_T values for each strain of the respective target species. Representative data used in these regressions are illustrated in Fig. 1 for *C. albicans*. Parallelism of the regressions for each strain was tested by means of an F test on the marginal mean squares resulting from the extra sums of squares for the parallel model, in which a common slope parameter was used for all strains, compared to the error mean square of the nonparallel model, in which a unique slope parameter was estimated for each strain. In either case, the intercept parameters were allowed to vary among strains. Given no significant differences in the slopes of the strains within any of the target species (data not shown), parallelism across species was then evaluated in a similar manner, constraining the slope parameter to a single value for all species. Slight variations were seen in the amplification efficiencies of the different species (Table 3).

Extrapolation of the regression lines to a C_T value of 40 provided estimates of the minimum cell detection limits for each strain. In order to measure inherent experimental variability in these results, we performed duplicate experiments on five different strains within four of the studied *Candida* species. Results from these five duplicate sets were pooled to obtain an estimate of experimental variance. For each species, the variance across strains, as given by the mean squares among strains, was compared to the pooled experimental variance for the duplicate analyses via an F test in order to determine whether it may be reasonable to attribute any observed variability between strains to normal experimental variability. These analyses indicated that the differences in extrapolated detection limits seen among the strains in each species could be attributed to experimental variability.

Experimental analysis of method sensitivity and accuracy. Tests of the method with known numbers of target *C. albicans* cells from pure culture, as confirmed by solid-phase cytometry, demonstrated a high degree of sensitivity and accuracy in the quantitative measurements (Fig. 2). Extrapolation of the responses for spikes in the range of 1 to 100 cells indicated a mean minimum detection limit (probability of detection, 50%) of 1.2 cells. This was borne out by the low cell count observations, where two of four instances of a single cell on a filter were successfully detected by QPCR. These results also indi-

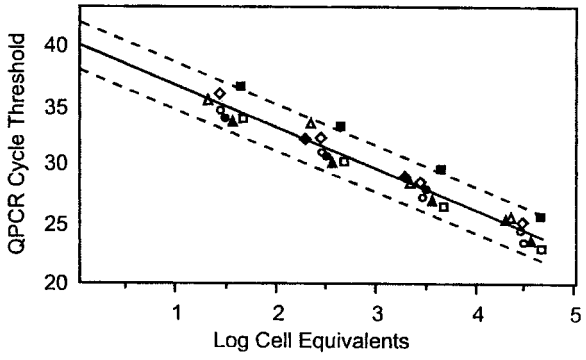

FIG. 1. Determination of amplification efficiency and extrapolated cell detection limit for QPCR analysis of *Candida* species, showing *C. albicans* as an example. DNA extracts were prepared by a rapid bead-milling method, as described in the text, from cell stocks of known concentration (determined by hemocytometer counts) of the strains ATCC 18804 (◇), ATCC11006 (◆), ATCC 14053 (▲), ATCC 60193 (△), ATCC 18804 (●), ATCC 24433 (○), ATCC 66027 (■) (experiment 1), and ATCC 66027 (□) (experiment 2). In this example, there was no lack of fit for a parallel relationship ($P = 0.729$), and thus the amplification efficiencies were presumed to be equal. The regression of log cell equivalents on C_T is represented by a single solid line. The dashed lines represent the 95% confidence limits for the individual results.

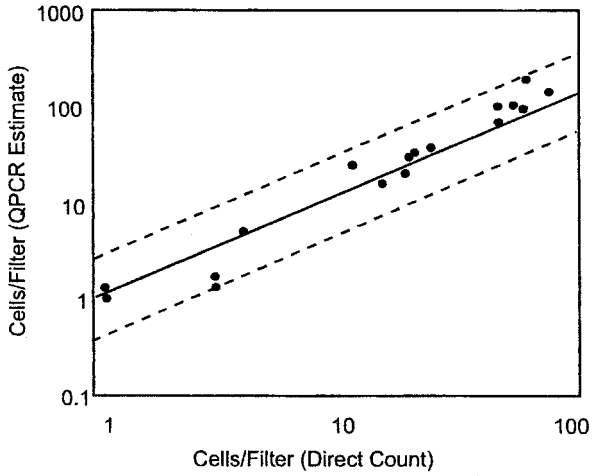

FIG. 2. Experimental sensitivity and accuracy of the CFE and QPCR analysis method for quantifying *Candida* cells. Known numbers of fluorescently labeled *C. albicans* cells were collected by filtration on polyethylene terephthalate filters and corroborated by solid-phase cytometry and microscopy. The counted cells on the filters were extracted in the presence of *G. candidum* reference cells by using the CFE method, and *C. albicans* cells were quantified by QPCR and $\Delta\Delta C_T$ comparative C_T analyses. The regression of log cell numbers per filter determined by direct counts on log cell numbers determined by QPCR analysis is represented by a single solid line. The dashed lines represent the 95% confidence limits for the individual results.

cate that 95% of the time, the estimate obtained from a single analysis will be ±0.3 logs (i.e., from 50 to 200%) of the true value. When triplicate analyses are utilized, as recommended, the estimate can be expected to range from 67 to 150% of the true value.

Tap, pond, and river water samples were used to determine the performance of the method for measuring pathogenic *Candida* species in different backgrounds. The results in Table 4 show that *Candida* cells can be sensitively and accurately enumerated in 1 liter of tap water by QPCR analysis by using the CFE method with minimal dilution of the DNA extracts and with no additional purification. In contrast, extracts of the pond and river water samples required additional dilution or purification (CFE+Q method) of the DNA extracts to obtain accurate results (Table 4).

Analysis of Lake Michigan beach samples. The weekly sampling of the recreational beach water from Lake Michigan showed that the cell quantities of the target organisms were generally low as determined both by QPCR analysis and live culturing (Table 5). As with the previously analyzed surface water samples, it was necessary either to dilute the DNA extracts from the majority of these samples or to subject them to further purification. Estimated cell detection limits, based on *G. candidum* reference assay results for the different samples and reference and target assay results for the equivalently processed and diluted calibrator samples, ranged from 5 to 40 cells in QPCR assays where no target cells were detected (Table 5). CFU of different target species were obtained in a number of these instances where corresponding QPCR results were negative; however, the quantities of CFU were below the estimated sensitivity range of the QPCR assays in nearly all of these cases. On the other hand, when target cells were detected in the samples by QPCR, they were generally found in substantially higher numbers than by plate counts. As a result, the estimates of total *Candida* cells across all targeted species for all samples were significantly higher by the QPCR method than by the plates counts ($P < 0.01$), with a median QPCR-to-CFU ratio of about 5.0. There was a positive correlation ($R = 0.91$ based on log counts) between the QPCR results for total target *Candida* and total colony counts of these species (Fig. 3). A positive correlation ($R = 0.93$) was also seen between the total cell quantities of the target *Candida* species detected in the QPCR analyses and colony counts of *Enterococcus* (Fig. 4). PCR amplicons generated by species-specific primer sets from two of the water samples were subjected to nucleotide sequence analyses and in each case showed complete agreement in callable bases with the published sequences of the corresponding target organisms.

DISCUSSION

Guiver et al. (16) developed a qualitative clinical method for identifying pathogenic species of *Candida* based on TaqMan chemistry. However, for environmental analysis of water, it is

TABLE 4. Enumeration of *C. albicans* cells added to different water samples by QPCR by using different sample preparation methods and extract dilutions

Sample type	Turbidity (NTU)[a]	Sample volume (l)	Cells added to sample[b]	Extract prep. method	Extract dilution	Measured cells/ sample[c]
Tap	2	1	90	CFE[d]	1:1	163
			20			35
			6			4
			0			<1[e]
Tap	2	1	90	CFE+Q[f]	1:1	123
			20			30
			6			4
			0			<1
Pond	20	0.1	1,000	CFE	1:10	1,237
			0			2[g]
Pond	20	0.1	1,000	CFE+Q	1:1	692
			0			1[g]
River	200	0.02	90	CFE	1:100	93
			20			30
			6			<22
			0			<37
River	200	0.02	90	CFE+Q	1:10	335
			20			32
			6			10
			0			<19

[a] NTU, nephelometric turbidity units.
[b] Arithmetic mean for three to five replicate analyses, estimated from solid-phase cytometry.
[c] Geometric mean for three to six replicate samples, estimated from QPCR analysis.
[d] CFE, collection filter extraction.
[e] <, no target detection: $C_T = 40$. Accompanying values are minimum cell detection limits determined by using this C_T result, the reference C_T results of the test samples, and target and reference C_T results of the corresponding calibrator samples in the $\Delta\Delta C_T$ calculation.
[f] CFE+Q, collection filter extraction with the added step of a Qiagen kit purification.
[g] Presumptive indigenous target organisms in the water sample.

TABLE 5. Cellular enumeration of organisms of six pathogenic *Candida* species in 100- to 300-ml Lake Michigan beach water samples by culture plate counts coupled with QPCR identification of colonies and by direct QPCR analysis[a]

Sample date	Turbidity (NTU)	Analysis method	No. of organisms of the *Candida* species:						Total
			Calb	Ckru	Cpar	Cglab	Ctrop	Clus	
8/13/01	10	Culture	9	108	0	0	14	4	135
		PCR[b]	11	312	38	9	11	40	421
8/20/01	8	Culture	1	10	0	0	1	0	12
		PCR[c]	<10[d]	61	<28	<11	<8	<8	61
8/27/01	19	Culture	0	4	0	7	5	0	16
		PCR[c]	<13	46	<29	<12	<8	<5	46
9/04/01	23	Culture	1	5	1	5	0	0	12
		PCR[c]	13	147	<16	<7	7	<5	167
9/11/01	37	Culture	–[e]	–	–	–	–	–	–
		PCR[f]	<13	64	<38	<15	20	<16	84
9/17/01	5	Culture	0	1	0	1	0	0	2
		PCR[f]	<6	<14	<20	<8	10	<5	10
9/24/01	82	Culture	1	4	3	4	2	1	15
		PCR[f]	<13	62	<40	<18	<12	<12	62

[a] Mean results from three replicate samples based on 100-ml sample volumes for 9/24/01; all others are based on 300 ml.
[b] Extract prepared with collection filter extraction (CFE); no dilution of extract for analysis.
[c] Extract prepared with collection filter extraction (CFE); 1/10 dilution of extract for analysis.
[d] <, no target detection: $C_T = 40$. Accompanying values are minimum cell detection limits determined by using this C_T result, the reference C_T results of the test samples, and target and reference C_T results of the corresponding calibrator samples in the $\Delta\Delta C_T$ method.
[e] –, not determined because of disruption in shipping.
[f] Extract prepared by collection filter extraction plus Qiagen kit purification (CFE+Q); no dilution of extract for analysis.

critical to develop not only specific assays but also highly quantitative and sensitive assays and the method to use them. As described in the present report, this has now been done.

The assays developed in this study target the variable D1/D2 domain of the LSU ribosomal gene and were highly specific when experimentally tested against a wide variety of related and unrelated organisms. The availability of an extensive database of yeast sequences for the LSU target region and associated phylogenetic analyses of these organisms (24) also allowed testing of the primers by computer analysis against closely related species for which no cultures were available and further supports our confidence in the specificity of these assays.

As implied by the extrapolation results in Table 3 and demonstrated by the experimental results in Fig. 2, the method provides sensitivities of detection approaching one cell per filter for pure cultures with no PCR inhibitors present. Results from Table 4 suggest that a similar level of sensitivity can be expected for the analyses of 1-liter tap water samples by this method, which will be critical for the analysis of drinking water, as opposed to clinical samples, where high populations would typically be found. Our results further indicate that the accuracy of the method provides a 95% confidence range from 50 to 200% of the true value in such relatively PCR inhibitor-free water samples.

Results from Tables 4 and 5, not surprisingly, suggest that the sensitivity of the method decreases roughly in proportion to the extent to which DNA extracts from various surface water samples must be diluted to overcome PCR inhibition. The suspended solid content (turbidity) of these water samples largely dictated the volumes that could be filtered and hence analyzed at one time. For most samples, either no dilution or a tenfold dilution of the DNA extract was sufficient for analysis. Some samples, generally those of higher turbidity (e.g., the river water sample in Table 4), required 100-fold dilution

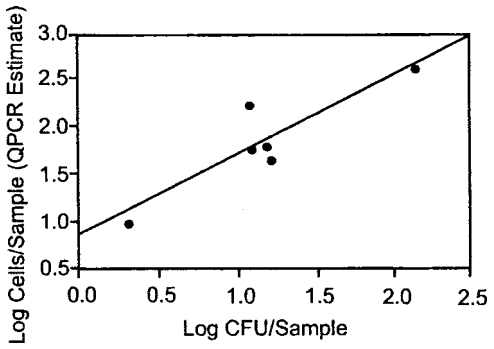

FIG. 3. Relationship between cellular enumeration of target *Candida* species in Lake Michigan beach water samples by culture plate counts (CFU) and by QPCR analysis. Water samples were filtered on nitrocellulose filters for plate counts on BiGGY agar medium, and the resulting CFU of target *Candida* species were enumerated as described in Table 5 and in the text. Equivalent water sample volumes were filtered through polycarbonate filters, the filters were extracted in the presence of *G. candidum* reference cells, and the extracts were diluted or further purified as indicated in Table 5. Quantities of each of the six *Candida* populations were determined by using QPCR assays and $\Delta\Delta C_T$ comparative C_T analyses. Results are shown for the six water samples indicated in Table 5 for which both methods of analysis were performed and are based on the total cell numbers of the six pathogenic *Candida* species, as determined by each method for each sample. The regression value for the line is $R = 0.91$.

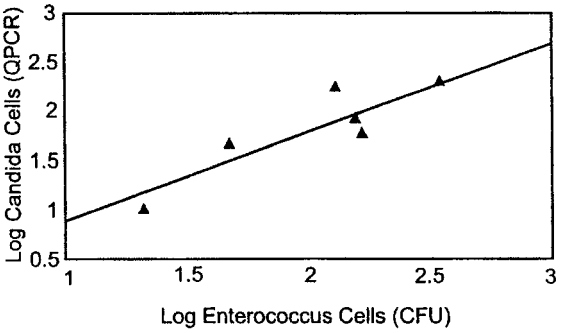

FIG. 4. Relationship between cellular enumeration of *Enterococcus* species by culture plate counts (CFU) and target *Candida* species by QPCR analysis. Water samples were filtered on nitrocellulose filters for plate counts on mEI basal medium, and the resulting colonies with blue halos were enumerated as presumptive enterococcal CFU. Results are presented on the basis of a 100-ml water sample volume. Quantities of each of the six *Candida* populations were determined as described in the legend for Fig. 3, with results presented on the basis of a 300-ml water sample volume. The regression value for the line is $R = 0.93$.

or additional purification of the extract. We further observed that purification of the DNA extracts could result in an almost tenfold loss of DNA in some instances, as demonstrated by increases in both the target and reference assay C_T values from calibrator extracts following this procedure. Such variations in DNA recovery will also affect the method's sensitivity. As a consequence, the sensitivity of this method can vary appreciably with different surface water samples and in some instances may not be as great as by culturing. Nevertheless, a strong correlation was observed between total plate counts of the target species and total numbers of these organisms as determined by QPCR analysis in this study. Also of interest was the observation that the QPCR method gave significantly higher overall estimates of target cell numbers than the plating method across all samples and species. Further studies will be needed to determine whether this observation can be attributed to low culturability of these organisms from the environment or possibly to their heterogeneous distribution in the water as the result of cell clumping or their occurrence in multicellular structures. While PCR template contamination of the extracts could also potentially contribute to these results, negative controls in these experiments consistently gave no signals in parallel analyses.

The QPCR method may be highly useful for detecting pathogenic *Candida* in both potable and surface waters as these and other fungi become more of a nosocomial and environmental risk. It has been known for many years that yeasts like *Candida* occur in drinking water (4, 7, 8). Rosenzweig et al. (30, 31) showed that various fungi, including yeasts, were relatively resistant to chlorine inactivation and survived conventional water treatment. This survival can lead to their accumulation in distribution systems. For example, densities of *C. parapsilosis* ranged from 3.1 to 4.6 CFU/cm^2 of surface area in the Springfield, Mo., water distribution system (12). Preliminary results from our laboratory with a fluorescently labeled *C. albicans* antibody and solid-phase cytometry have suggested the presence of pathogenic *Candida* species in U.S. tap water (N. E. Brinkman, R. A. Haugland, J. W. Santo Domingo, and S. J. Vesper, Abstr. 101st Gen. Meet. Am. Soc. Microbiol. 2001, abstr. Q-417, p. 669).

Candida has also been isolated from drinking water outside the U. S. In France, 50% of samples were contaminated with yeasts, with *Candida* being most common (22). In Finland, 38 samples of chlorinated drinking water were examined. Yeasts, mostly *Candida* species, occurred in 50% of the samples (27). In Greece, *Candida* species were isolated from the hospital water supply and from hemodialysis units (2, 3).

Recreational waters may also pose a threat to human health from candidiasis (13, 14). *Candida* species are frequently isolated from human-impacted surface water and sewage (11, 40). Bergen and Wagner-Merner (5) isolated *Candida* spp. from beaches in Tampa Bay, Fla. A study in the 1970s of the river water entering Lake Michigan just west of Mount Baldy recreational beach showed that *C. krusei* was the most numerous *Candida* species (40). Our results show that *C. krusei* is still the most numerous of the pathogenic yeast in this environment. Besides the direct analysis of pathogenic *Candida* like *C. krusei*, this technology may also be useful as a method to monitor fecal pollution and changing populations.

Bacterial indicators of fecal pollution are sometimes confounded because of source tracking issues. Quantification of *Candida* may provide an alternative or additional indicator of fecal pollution. Another use might be as an early warning system for change in a water supply by comparing ratios of *Enterococcus* to *Candida* populations over time. Also, as the occurrence of drug-resistant yeasts increases, the monitoring of the changes in yeast populations of fecal origin may become important.

A significant advantage of QPCR technology over culturing lies in its combination of speed and accuracy. Sample processing can be completed within approximately to 30 to 90 min, depending on whether the CFE or CFE+Q sample extraction method is used, and sample analysis times can range from approximately 30 min to 2 h, depending on the choice of thermal cycling instrument (10). This is opposed to the 48 h required for culturing methods. In cases like a bathing beach, significant spikes of pathogenic *Candida* species resulting from untreated effluents or storm runoff could be quickly detected. This method for the analysis of surface water could be used as a practical tool for rapid, same-day communication for risk assessment and possible use for beach closure deliberations. Protection of drinking water supplies also requires timely and specific information about the species and quantities of microbial pathogens, which this technology can provide.

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