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Rapid Identification of up to Three *Candida* Species in a Single Reaction Tube by a 5′ Exonuclease Assay Using Fluorescent DNA Probes

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We used fungus-specific PCR primers and species-specific DNA probes to detect up to three *Candida* species in a single reaction tube by exploiting the 5′ to 3′ exonuclease activity of *Taq* DNA polymerase. Probes to the internal transcribed spacer region of the rRNA gene were labeled at the 5′ end with one of three fluorescent reporter dyes, 6-carboxy-fluorescein (FAM), tetrachloro-6-carboxy-fluorescein (TET), or hexachloro-6-carboxy-fluorescein (HEX), and at the 3′ end with a quencher dye, 6-carboxy-tetramethyl-rhodamine. During PCR amplification, each reporter dye emits a characteristic wavelength as it is cleaved from its specific target DNA and from the quencher dye. Therefore, signals from up to three probes can be detected simultaneously during the PCR assay. Six probes were designed for use in this study: CA-FAM, CT-TET, and CP-HEX were added to one tube to simultaneously detect the typically fluconazole-sensitive species *C. albicans*, *C. tropicalis*, and *C. parapsilosis*, respectively. CG-FAM and CK-TET were added to a second tube to simultaneously detect the typically more innately fluconazole-resistant species *C. glabrata* and *C. krusei*, respectively. All-CAN-TET, a *Candida* genus probe, was added to a third tube to detect DNAs from all *Candida* species tested. DNAs recovered from 61 blood culture bottles, including 23 positive for *C. albicans*, 18 positive for *C. glabrata*, 6 positive for *C. tropicalis*, 6 positive for *C. krusei*, 5 positive for *C. parapsilosis*, and 3 positive for mixed fungemias, were tested. Control samples included those from blood culture bottles with no growth ($n = 10$) or from patients with confirmed bacteremia ($n = 10$). Probes detected and correctly identified the organisms in 58 of 61 specimens (95.1%) and gave no false-positive results. This method is simple and rapid and does not require post-PCR hybridization and incubation steps. It is sensitive and specific for the detection and identification of *Candida* species from blood culture bottles, including those containing mixtures of *Candida* species, and should facilitate an earlier specific diagnosis, leading to more appropriately targeted antifungal drug therapy.

Rapid identification of *Candida* species has become particularly important because of the increase in the numbers of infections caused by newly emerging species (1, 2, 31, 32). Although not conclusively determined, it has been suggested that this increase in infections caused by more innately fluconazole-resistant *Candida* species such as *C. glabrata* (18, 33) and *C. krusei* (31, 32) may be a direct result of the widespread use of fluconazole for the prophylaxis and treatment of candidiasis (18, 20, 33). Rapid species identification is therefore required for appropriate targeting of antifungal drug therapy and an optimal therapeutic outcome (18, 20, 22, 24).

Whereas *C. albicans* can be presumptively identified by germ tube formation tests, *C. albicans*, *C. krusei*, and *C. tropicalis* can be presumptively identified by growth on CHROMagar medium, and other species of *Candida* can be identified by rapid (4-h) enzymatic tests, each of these procedures requires that the organism be grown on solid medium for at least 24 h and, more often, for 48 h before such tests can be performed or their results interpreted (19, 29). In addition, not all *C. albicans* isolates form germ tubes, and the newly described species, *C. dubliniensis*, which has been reported to develop a drug-resistant phenotype more rapidly than *C. albicans*, is germ tube and chlamydospore positive (26). The "gold standard" for definitive yeast identification requires further analysis by assimilation and fermentation tests, which can require up to 28 days to complete (29). Therefore, a definitive test for the rapid identification of *Candida* isolates to the species level would be clinically and epidemiologically important.

We previously described a clinically useful PCR-based method for the rapid detection and identification of *Candida* species isolates from positive blood culture bottles (25). This consisted first of a simple DNA extraction procedure with heat, detergent, and mechanical breakage of cells that did not require the use of expensive enzymes or phenol-chloroform. A simple, rapid, and sensitive microtitration plate format and colorimetric detection of digoxigenin-labeled species-specific probes were then used (9, 25). Here we describe a more rapid method in which a 5′ exonuclease assay and fluorescent DNA probes are used (10, 14). The method can be used to differentiate simultaneously up to three *Candida* species in a single reaction tube.

Together with the increased specificity afforded by this method (i.e., unless the probe binds to a specific, complementary target DNA, no signal will be generated), this method reduces the PCR cycling time by using two-step PCR cycling rather than traditional three-step PCR cycling and eliminates additional post-PCR hybridization by using fluorescent DNA probes that anneal to the target DNA during the amplification step. Thus, the total reaction time required for definitive spe-

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TABLE 1. Probes used for fluorescence detection of DNA

Probe	Nucleotide sequence (5′ to 3′) and chemistry[a]
All-CAN-TET	5′-TET AG GGC ATG CCT GTT TGA GCG TC(GA) TT-3′-P
CA-FAM	5′-FAM AT TGC TTG CGG CGG TAA CGT CC-3′-P
CT-TET	5′-TET CA AAA CGC TTA TTT TGC TAG TGG CC-3′-P
CP-HEX	5′-HEX GG TAC AAA CTC CAA AAC TTC TTC CA-3′-P
CG-FAM	5′-FAM TA GGT TTT ACC AAC TCG GTG TT GAT-3′-P
CK-TET	5′-TET AG TGG CCC GAG CGA ACT AGA CTT TT-3′-P

[a] Each probe also contained a quencher dye, TAMRA, attached to a linker arm-modified nucleotide near its 3′ end.

cies identification is reduced to 5 h by the 5′ exonuclease method.

MATERIALS AND METHODS

Clinical samples. A total of 81 samples from blood cultured in BacT/Alert bottles (Organon Teknika Corporation, Durham, N.C.) were tested: 61 for patients with candidemia, 10 for patients with bacteremia, and 10 for patients whose bottles had no growth. Twenty milliliters of blood from each patient with suspected bacteremia or fungemia was collected at the bedside, and 10 ml was immediately inoculated into each aerobic and anaerobic BacT/Alert bottle for culture. The inoculated bottles were agitated continuously in the BacT/Alert instrument (Organon Teknika Corporation) at a rate of 68 cycles per min and were incubated at 35 to 37°C for 5 days or until the bottles were positive by colorimetric detection of CO_2. Aliquots from positive bottles were Gram stained and subcultured. Bottles suspected of containing *Candida* spp. by Gram staining were selected, and 2-ml aliquots were removed and stored at −30°C. During the study period, *Candida* spp. were isolated from 61 blood culture bottles containing samples from 24 patients. Of 61 bottle sets from which *Candida* spp. were isolated, *C. albicans* blastoconidia were isolated from 23 bottles, *C. glabrata* was isolated from 18 bottles, *C. tropicalis* was isolated from 6 bottles, *C. krusei* was isolated from 6 bottles, *C. parapsilosis* was isolated from 5 bottles, and both *C. glabrata* and *C. albicans* were isolated from 3 bottles. Isolates were identified by conventional sugar fermentation and assimilation tests and by germ tube and chlamydospore formation (29). Ten randomly selected samples from patients with bacteremia due to coagulase-negative *Staphylococcus* ($n = 2$), *Enterococcus* spp. ($n = 2$), *Citrobacter freundii* ($n = 2$), *Corynebacterium* spp. ($n = 2$), or a mixture of *Enterococcus* spp. and *Staphylococcus aureus* ($n = 1$) or *Klebsiella pneumoniae* and *Acinetobacter calcoaceticus* ($n = 1$) were also tested as negative controls. Clinical specimens which never became positive during incubation ($n = 10$) were also tested as negative controls.

In addition to clinical samples, BacT/Alert bottles spiked with *C. albicans* B311 at 0, 10^1, 10^2, 10^3, 10^4, and 10^5 blastoconidia per 200 μl of rabbit whole blood were tested (broth to rabbit blood ratio, 8:1). The specificities of the DNA probes were also tested against purified DNA from various bacteria, other fungi, or a human placental cell line (9).

Extraction of DNA. A previously described mechanical disruption method (25) was used for DNA extraction. Briefly, 200 μl of sample was added to 800 μl of TXTE buffer (10 mM Tris, 1 mM EDTA [pH 8.0], 1% Triton X-100) in a sterile, 1.5-ml centrifuge tube, and the mixture was incubated for 10 min at room temperature. After lysis, cell debris and *Candida* blastoconidia were pelleted by centrifugation at 14,000 rpm for 5 min (centrifuge model 5403; Eppendorf, Engelsdorf, Germany). After three washes by centrifugation with 1 ml of TXTE buffer, the pellet was resuspended in 300 μl of TXTE buffer and the mixture was transferred to a 2-ml screw-cap, conical-bottom tube containing 200 μl of 0.5-mm-diameter zirconium beads (Biospec Products, Bartlesville, Okla.). After boiling for 15 min, the mixture was shaken for 20 min in a mechanical cell disrupter (Mini-Beadbeater; Biospec Products). After centrifugation for 20 s, the supernatant was stored at −20°C until it was used for PCR amplification.

Purified DNA. Purified DNAs from isolates of *C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. glabrata*, and *C. krusei* were used as template standards for each PCR. These DNAs and purified DNAs from other *Candida* species, *Saccharomyces cerevisiae*, *Cryptococcus neoformans*, *Aspergillus fumigatus*, *Aspergillus flavus*, *Penicillium marneffei*, *Histoplasma capsulatum*, *Blastomyces dermatitidis*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and a human placental cell line were obtained by mechanical breakage or enzymatic lysis followed by ethanol precipitation and phenol-chloroform extraction by conventional means as described previously (9, 15, 16). All strains of microorganisms used in the study except for *C. kefyr* WO696 were described previously (9, 25); *C. kefyr* was obtained from the Mycology Reference Laboratory Culture Collection, Centers for Disease Control and Prevention.

Fluorescent probe design and synthesis. Probes consisted of oligonucleotides labeled at their 5′ ends with one of three available fluorescent reporter dyes: 6-carboxy-fluorescein (FAM), tetrachloro-6-carboxy-fluorescein (TET), or hexachloro-6-carboxy-fluorescein (HEX) (10, 14). The probes also contained a quencher dye, 6-carboxy-tetramethyl-rhodamine (TAMRA), attached to a linker arm-modified nucleotide near the 3′ end and a 3′-blocking phosphate (10, 14). Reporter dye fluorescence was suppressed by the quencher dye during specific binding of the probe to complementary DNA. Fluorescence was then generated as the probe was cleaved by the 5′ exonuclease activity of the *Taq* polymerase during the PCR (10, 14). The six probes used in this study are listed in Table 1: All-CAN-TET for the detection of all the DNAs of *Candida* species and CA-FAM, CT-TET, CP-HEX, CG-FAM, and CK-TET for the detection of *C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. glabrata*, and *C. krusei* DNAs, respectively.

PCR assay. The PCR assay was performed with the universal fungal primers ITS3 and ITS4 (9, 30) and by a standard PCR protocol (9) modified by the addition of fluorescent probes (10, 14). Based upon the guanine-plus-cytosine content, the predicted melting temperatures (T_ms) of the probes for all *Candida* spp. and *C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. glabrata*, and *C. krusei* were 80, 70, 70, 70, 76, and 72°C, respectively. On the other hand, the T_ms of primers ITS3 and ITS4 were 62 and 58°C, respectively. Therefore, the probes were redesigned from those reported previously (9, 25) to optimize primer extension and to allow multiple probes to bind with similar frequencies when they are mixed in one reaction tube (Table 1). PCR was performed with a 1-μl sample in a total volume of 50 μl containing 10 mM Tris-HCl, 50 mM KCl (pH 8.3), 3.5 mM $MgCl_2$, dATP, dGTP, dCTP, and dTTP each at a concentration of 0.2 mM, each primer (ITS3 and ITS4) at a concentration of 0.2 μM, 2.5 U of *Taq* DNA polymerase (Boehringer Mannheim, Indianapolis, Ind.), and one, two, or three fluorescent probes (final concentrations, 10 to 50 nM). A two-step PCR with a combined annealing and extension step was performed in a 9600 thermocycler (Perkin-Elmer, Emeryville, Calif.). All cycles began with a DNA denaturation step for 5 min at 94°C. After this, cycles consisted of 30 s at 95°C (denaturation) and 1 min and 30 s at 58°C (annealing and extension) for 40 cycles. Primer extension at 72°C for 10 min followed the final cycle.

Negative controls (no template DNA) were tested by using the same reaction mixture under the amplification conditions described above but without template DNA. Positive standards for PCR used 1 ng of purified DNA for each *Candida* species to be detected.

Quality control. Each reaction was carried out in duplicate or triplicate. One nanogram each of *C. albicans* and *C. glabrata* DNA was used as a positive control for each sample run. Carryover contamination was reduced by using aerosol-resistant pipet tips and separate laboratory areas for DNA sample preparation and PCR amplification along with other standard contamination precautions (9, 13).

Detection of PCR amplicon fluorescence. Immediately following the last PCR cycle or after storage at 4°C until the next day, 40 μl from each PCR tube was transferred to a white, 96-well microtitration plate (Dynatech, Chantilly, Va.). Forty microliters of TE buffer (10 mM Tris, 1 mM EDTA [pH 8.0]) was used as a buffer blank. The plate was then read on a LS 50B Luminescence Spectrometer (Perkin-Elmer, Applied Biosystems, Inc., Foster City, Calif.) with a microtitration plate reader attachment. An excitation wavelength of 488 nm was used and the emission wavelengths for the reporter dyes were as follows: FAM, 518 nm; TET, 538 nm; and HEX, 556 nm. The emission wavelength for the quencher dye (TAMRA) was 582 nm. A fluorescent data management system that uses EXCEL-compatible macros was used for data analysis (Perkin-Elmer, Applied Biosystems, Inc.).

Data analysis and interpretation. Using the TaqMan data worksheet and macro (Perkin-Elmer, Applied Biosystems, Inc.), the delta RQ for each sample was automatically calculated. The delta RQ is defined as an increase in the emission intensity ratio of the reporter dye after release from the quencher dye on the fluorescent probe (RQ+) minus the baseline emission intensity of the quenched reporter dye on the intact fluorescent probe (RQ−), or delta RQ = (RQ+) − (RQ−), where RQ+ (PCR with target DNA) is equal to emission intensity of reporter with DNA/emission intensity of quencher with DNA and RQ− (PCR without target DNA) is equal to emission intensity of reporter without DNA/emission intensity of quencher without DNA. A threshold RQ is calculated to ensure a statistically high confidence level (99%) when the standard deviation (SD) obtained from triplicate, no-DNA-template controls is used.

We used the multicomponent data program to interpret PCR results when using multiple probes simultaneously. The multicomponent data program automatically displayed the results as "no DNA," DNA template 1, DNA template 2, or DNA template 3 when either the TaqMan 3 Allele-Genotype Worksheet or the two-reporter multicomponent worksheet for the well plate reader software

TABLE 2. Detection and identification of *Candida* species in positive blood cultures with multiple fluorescent probes[a]

Blood culture result	Delta RQ values for the following PCRs with the indicated probes:					
	PCR A			PCR B		PCR C (All-CAN-TET)
	CA-FAM	CT-TET	CP-HEX	CG-FAM	CK-TET	
C. albicans ($n = 23$)	0.950 ± 0.129	0	0	0	0	3.418 ± 0.140
C. tropicalis ($n = 6$)	0	0.483 ± 0.156	0	0	0	1.920 ± 0.557
C. parapsilosis ($n = 5$)	0	0	0.330 ± 0.053	0	0	1.784 ± 0.671
C. glabrata ($n = 18$)	0	0	0	0.630 ± 0.091	0	2.806 ± 0.291
C. krusei ($n = 6$)	0	0	0	0	0.727 ± 0.136	3.377 ± 0.378
No growth ($n = 10$)	0	0	0	0	0	0
Bacteremia ($n = 10$)	0	0	0	0	0	0

[a] Values are expressed as means ± standard errors (see Materials and Methods). Means ± standard error delta RQ values for all negative control samples were as follows: for the CA-FAM probe ($n = 55$), 0.021 ± 0.003; for the CT-TET probe ($n = 72$), 0.033 ± 0.006; for the CP-HEX probe ($n = 73$), 0.052 ± 0.008; for the CG-FAM probe ($n = 60$), 0.009 ± 0.001; for the CK-TET probe ($n = 72$), 0.025 ± 0.004; for the All-CAN-TET probe ($n = 20$), 0.060 ± 0.014. The P value was <0.01 for positive test samples versus negative control samples for all probes.

was used (Perkin-Elmer, Applied Biosystems, Inc.). The "no DNA" threshold was automatically calculated from values 2 SDs above the mean for the negative controls (value = 1.00). We established the cutoff value for a positive probe result as 2 SDs above the mean delta RQ for negative control values in PCRs with multiple probes. That is, in PCR A, a positive result was defined as a delta RQ above 0.066 (2 SDs above the mean for the negative controls) for the CA-FAM probe, 0.126 for the CT-TET probe, and 0.190 for the CP-HEX probe. Similarly, in PCR B, a positive result was defined as a delta RQ above 0.034 (2 SDs above the mean for the negative controls) for the CG-FAM probe and above 0.078 for the CK-TET probe. For the All-CAN-TET probe, which detects all *Candida* species and gives a significantly higher value relative to those given by the species-specific probes, we used a cutoff higher than the one that we used for the individual species probes. A value of 0.308 (4 SDs above the mean delta RQ for all negative controls tested) was used as the positive cutoff value for this probe.

Statistical analysis. Student's t test was used to establish differences between test groups. A P value of ≤0.05 was considered significant.

RESULTS

Detection and identification of *Candida* species in blood culture bottles using 5′ exonuclease PCR and multiple fluorescent probes. The fluorescent probes used in this study are described in Table 1. Probe sequences were modified from those described previously for the colorimetric detection of *Candida* species DNA (9, 25) in order to optimize T_ms and thereby allow multiple probes to anneal to their respective target DNAs with similar efficiencies. In addition, an all-*Candida* genus probe (All-CAN-TET) was designed to detect all *Candida* species tested.

The PCR assay could therefore use up to three fluorescent probes, with the same excitation wavelength but different emission wavelengths, simultaneously in one reaction tube. In this manner, we could group the traditionally azole-sensitive species (*C. albicans*, *C. tropicalis*, and *C. parapsilosis* using CA-FAM, CT-TET, and CP-HEX probes, respectively) to be identified in one reaction tube (PCR A) and the traditionally more azole-resistant *Candida* species (*C. glabrata* and *C. krusei* using CG-FAM and CK-TET probes, respectively) to be identified in the second reaction tube (PCR B) and then use an all-*Candida* genus probe (All-CAN-TET) to simultaneously detect all *Candida* species tested in a third reaction tube (PCR C).

As shown in Table 2, fluorescent probes were highly specific for the identification of the appropriate *Candida* species. *C. albicans*, *C. tropicalis*, and *C. parapsilosis* were each correctly identified in PCR A, *C. glabrata* and *C. krusei* were correctly identified in PCR B, and all *Candida* species were detected by the All-CAN-TET probe in PCR C. No cross-reactions with other *Candida* species or with samples from patients with bacteremia or with samples from blood culture bottles with no growth were observed (Table 2). Despite the coexistence of bacteria with *Candida* in five of the blood culture bottles from patients with candidemia (including *Enterococcus* spp. [$n = 4$] and coagulase-negative *Staphylococcus* [$n = 1$]), no interference with the PCR detection of *Candida* species was observed.

Two of the three samples from patients with mixed candidemias were identified by PCR to contain both *C. glabrata* and *C. albicans*, which was in agreement with the results of conventional phenotypic identification methods. One culture, identified by conventional phenotypic methods as containing both *C. glabrata* and *C. albicans* (Table 3), was identified as containing only *C. glabrata* by PCR. However, the patient from whom the sample for culture was obtained had multiple blood samples that were culture positive for *C. glabrata* but only one blood sample that was culture positive for *C. albicans*. It is likely, therefore, that the single positive *C. albicans* culture represents a contaminant that entered the sample during routine phenotypic identification procedures and was defined as such for this study.

Three cultures (one each of *C. glabrata*, *C. tropicalis*, and *C. parapsilosis*) gave values below the positive cutoff established for each of the corresponding fluorescent probes and were therefore considered to be falsely negative. No bacteria were recovered from these samples, and therefore, the false negativity could not be explained by interference from coexisting bacteria. These same cultures had been previously tested by the colorimetric PCR-enzyme immunoassay (PCR-EIA) (25) and were correctly identified as positive in that system.

Thus, probes for *C. albicans*, *C. tropicalis*, and *C. parapsilosis* (PCR A) and those for *C. glabrata* and *C. krusei* (PCR B) rapidly and correctly identified the isolates in 58 (95.1%) of 61 clinical blood cultures and the All-CAN-TET probe (PCR C) detected 100% of all *Candida* species in cultures, including cultures of samples from patients with mixed candidemias. The

TABLE 3. Routine versus fluorescent probe identification of *Candida* species in blood culture bottles

Routine identification	No. of bottles	Fluorescent probe identification	No. (%) of bottles
C. albicans	23	*C. albicans*	23
C. glabrata	18	*C. glabrata*	17
C. krusei	6	*C. krusei*	6
C. tropicalis	6	*C. tropicalis*	5
C. parapsilosis	5	*C. parapsilosis*	4
C. glabrata + *C. albicans*	3	*C. glabrata* + *C. albicans*	2
		C. glabrata only	1
Total	61		58 (95.1)

TABLE 4. Specificity of the All-CAN-TET fluorescent probe

Genomic DNA tested	Delta RQ values[a]
C. kefyr	4.843 ± 0.164[b]
C. parapsilosis	4.587 ± 0.159[b]
C. krusei	4.560 ± 0.377[b]
C. albicans	4.361 ± 0.104[b]
C. glabrata	3.330 ± 0.183[b]
C. tropicalis	3.273 ± 0.018[b]
S. cerevisiae	2.270 ± 0.175[b]
C. guilliermondii	2.180 ± 0.085[b]
A. fumigatus	1.661 ± 0.180[b]
A. flavus	1.132 ± 0.181[b]
H. capsulatum	0.210 ± 0.036
P. aeruginosa	0.187 ± 0.063
E. coli	0.177 ± 0.047
C. neoformans	0.173 ± 0.061
P. marneffei	0.160 ± 0.051
Human cell line	0.117 ± 0.093
B. dermatitidis	0.111 ± 0.110
S. aureus	0.060 ± 0.046

[a] Values are means ± standard errors. Data are for triplicate samples from one to three experiments.

[b] Positive value based on cutoff of 4 SDs above the mean for negative (no-DNA) control samples (mean ± SD delta RQ value for negative controls [n = 20], 0.060 ± 0.062; 4 SDs above the mean ± 0.308 cutoff for positivity).

sensitivity and specificity for the correct detection of the appropriate *Candida* species from blood cultures for PCR A (azole-sensitive species) were 91.9 and 100%, respectively; those for PCR B (azole-resistant species) were 96.3 and 100%, respectively; and those for PCR C (all *Candida* species) were 100 and 100%, respectively.

Specificity of the probe for all *Candida* species versus specificities of probes for other fungi. The All-CAN-TET probe was further tested to determine its capacity to detect other *Candida* species DNA without cross-reacting with other yeasts or fungi. The All-CAN-TET probe reacted with purified DNA from all *Candida* spp. tested, *S. cerevisiae*, *A. fumigatus*, and *A. flavus*, but it did not react with any other fungal, bacterial, or human DNA tested (Table 4). The lower values obtained for the detection of *Aspergillus* species DNA relative to those obtained for the detection of *Candida* species DNA were probably a reflection of sequence differences between these two genera in the target DNA region (6). Specific *Aspergillus* species and genus probes have now been developed to differentiate *Candida* species and other fungi from *Aspergillus* species (4, 6), and a probe for *S. cerevisiae* has been shown to be species specific in preliminary tests (8). These results have been reported separately (4, 6, 8).

Sensitivity of fluorescent probes for detection of known numbers of *Candida* blastoconidia. We compared the results obtained with fluorescent probes with those obtained by a colorimetric PCR-EIA method developed in our laboratory (9). *C. albicans* B311 blastoconidia were introduced at concentrations of 0, 10^1, 10^2, 10^3, 10^4, or 10^5 per 200 μl of BacT/Alert culture broth containing whole rabbit blood (broth to rabbit blood ratio = 8:1). The samples were then processed as described in Materials and Methods for fluorescence detection or as described previously for colorimetric and ethidium bromide detection of PCR products (9, 25). The results of this comparison are presented in Table 5.

The mean delta RQ value for the All-CAN-TET probe for each 200-μl sample was higher than that for the CA-FAM probe, as might be predicted from the results presented in Table 2. Although the signal strength for the All-CAN-TET probe was higher than that for the CA-FAM probe, the higher positive cutoff value used for the All-CAN-TET probe resulted in identical limits of sensitivity for both probes (i.e., 10^2 cells per 200-μl sample or 1 cell per 2-μl sample; $P < 0.01$ relative to the results for control samples containing no DNA). Similarly, although the signal strength for the colorimetric detection system probe (CA-DIG) was lower than that for either the All-CAN-TET or the CA-FAM probe, the cutoff value defined the limit of sensitivity to be identical to that for the fluorescent probes. In contrast, ethidium bromide staining of agarose gels for the detection of the PCR products was a log less sensitive than either the fluorescent probe assay or the PCR-EIA method (i.e., 10^3 cells per 200-μl sample) (Table 5).

DISCUSSION

We developed a rapid and simple 5′ exonuclease PCR method using fluorescent DNA probes to identify from blood culture bottles the five most medically important *Candida* species responsible for bloodstream infections. Species identification time was reduced from 7 h by colorimetric PCR-EIA to 5 h by the 5′ exonuclease assay. This was accomplished by elimination of the post-PCR hybridization step and the incubation step necessary for the colorimetric detection of the PCR product and by use of two-step rather than three-step PCR cycling. In addition, a generic *Candida* probe for the detection

TABLE 5. Comparative sensitivity of DNA detection by fluorescent probes, colorimetric PCR-EIA, and ethidium bromide staining

No. of *C. albicans* organisms per 200 μl	Mean ± SE delta RQ value with fluorescent probes[a,b]		Mean ± SE A_{650} by colorimetric PCR-EIA with CA-DIG[b]	Result by ethidium bromide staining of agarose gel
	All-CAN-TET	CA-FAM		
10^5	3.100 ± 0.263[c]	1.136 ± 0.231[c]	0.582 ± 0.029[c]	+
10^4	2.735 ± 0.104[c]	0.690 ± 0.058[c]	0.441 ± 0.035[c]	+
10^3	0.686 ± 0.069[c]	0.187 ± 0.017[c]	0.139 ± 0.017[c]	+
10^2	0.343 ± 0.040[c]	0.070 ± 0.012[c]	0.027 ± 0.006[c]	−
10^1	0.063 ± 0.017	0.013 ± 0.035	0	−
0	0	0	0	−
Purified DNA[d]	4.383 ± 0.098[c]	0.996 ± 0.046[c]	0.552 ± 0.058[c]	+

[a] Values are expressed as the mean ± standard error (SE) delta RQ values.

[b] Data are from three experiments, with duplicate wells used in each experiment.

[c] $P < 0.01$ or $P < 0.05$ by Student's t test versus the mean for samples containing no DNA. Positive cutoff values for the All-CAN-TET and CA-FAM fluorescent probes and for the CA-DIG colorimetric probe were 0.308, 0.066, and 0.015, respectively.

[c] One nanogram of *C. albicans* B311 DNA.

of all *Candida* species DNAs simultaneously in one reaction tube was developed.

Although others have described enzymatic lysis methods for the direct recovery of fungal DNA from whole blood (3, 7, 12), most require initial lysis of erythrocytes and leukocytes with salts, detergents, and/or proteinase K and multiple purification steps in order to obtain adequate DNA in sufficiently pure form for successful PCR amplification. Our laboratory also found that multiple purification steps were required for optimum PCR amplification of fungal DNA from whole blood (9). In order to circumvent the laborious, time-consuming, and hazardous (i.e., phenol-chloroform) steps required to obtain sufficient DNA from *Candida* cells recovered from whole blood, we chose to isolate *Candida* DNA from positive BacT/Alert blood culture bottles instead. This strategy allowed (i) inhibitors of PCR amplification to be diluted out by the BacT/Alert blood culture bottle medium, (ii) an additional "amplification" of DNA by the normal growth of *Candida* cells in the culture bottles, and (iii) the use of detergent, heat, and mechanical disruption for the isolation of *Candida* species DNA without the use of phenol-chloroform.

In addition, the method most commonly used to detect PCR amplicons is gel electrophoresis and ethidium bromide staining either with or without restriction enzyme digestion before visualization (3, 5, 11, 23). Southern blotting after gel electrophoresis adds another degree of sensitivity and specificity (12, 27) but is labor-intensive and can be hazardous and expensive if radioisotopically labeled probes are used. One investigator expanded on these techniques and used single-strand conformational polymorphisms to differentiate and detect fungal DNA (28). None of these methods, however, is easily adapted to the clinical setting. The fluorescent 5′ exonuclease assay, on the other hand, is rapid, simple to perform, sensitive, and specific and does not require a pure culture for correct species identification.

Probe mixtures were therefore designed for use in the 5′ exonuclease assay to discriminate typically fluconazole-sensitive *Candida* species (*C. albicans*, *C. tropicalis*, *C. parapsilosis*) from more innately fluconazole-resistant species (*C. glabrata*, *C. krusei*); this allowed a reduction in the number of sample manipulation steps that needed to be performed and the detection of up to three *Candida* species in a single reaction tube. Potentially, probe mixtures could be reformulated in any combination to accommodate the known resistance profiles at a particular institution, particularly now that regional differences in species distribution have been shown to occur (21).

The fluorescent, species-specific probes designed in this study detected and correctly identified *Candida* species DNA from 58 (95.1%) of 61 clinical blood culture bottles and gave no falsely positive results. Three samples that were positive by conventional methods and by colorimetric PCR-EIA were falsely negative by the fluorescent 5′ exonuclease assay. Falsely negative results were not caused by the coexistence of bacteria because these samples contained no bacteria (and in other samples, in which bacteria coexisted with *Candida* species, the fluorescent 5′ exonuclease assay was correctly positive). However, the probe sequences were slightly modified from those reported previously (9, 25) to optimize primer extension and to allow multiple probes to bind with similar frequencies when they were admixed in one reaction tube. Therefore, such slight modifications in the probe sequences may have resulted in reduced target detection in the three falsely negative samples. Alternatively, DNA samples were stored for longer times before being tested by the fluorescent 5′ exonuclease assay than by conventional methods or the colorimetric PCR-EIA (up to 5 months), and this longer storage may have resulted in some deterioration of the DNA target in these few samples. On the other hand, the all-*Candida* genus probe detected 100% of all *Candida* species DNA in all samples and did not cross-react with bacterial, human, or other fungal DNA with the exception of *S. cerevisiae*, *A. fumigatus*, and *A. flavus* DNA. Because the all-*Candida* genus probe was designed from the more conserved 5.8S region of the rRNA gene (15) rather than the less conserved ITS2 region, it is not surprising that some cross-reactivities were observed. However, with the sample preparation methods used in the present study, it is unlikely, although not impossible, that DNA from *A. fumigatus* or *A. flavus* would be recovered from blood cultures (the filamentous fungi are more resistant to breakage by the methods that we used [see reference 17 for a review]). Also, the values obtained for samples containing *Aspergillus* species DNA were much lower relative to those obtained for samples containing *Candida* species DNA and probably reflect the divergence in DNA sequence for these two genera in this region (4, 6). If the differentiation of *Candida* species from cross-reacting *Aspergillus* species and *S. cerevisiae* should be needed, however, preliminary studies have shown that a probe designed in our laboratory to differentiate *S. cerevisiae* from *C. glabrata* is species specific (8), and generic and species-specific probes have also been designed to detect and differentiate *A. fumigatus* and *A. flavus* from yeasts and other fungi (4, 6).

Currently, probe mixtures can be customized to identify up to three *Candida* species in a single reaction tube. If additional fluorescent dyes that have similar excitation wavelengths but different emission wavelengths from those used in the present study are developed, possibly even greater numbers of probes can be designed to detect more than three species in a single reaction tube (i.e., ideally, the simultaneous detection and differentiation of the five major *Candida* species). Also, the advent of more broad-spectrum antifungal agents (i.e., voriconazole), if proved to be clinically efficacious against all medically important *Candida* species, may allow a single determination with the all-*Candida* generic probe to suffice (at least until evidence of voriconazole resistance occurs). At present, this assay significantly reduces the time required to identify definitively the *Candida* species obtained from blood culture bottles and should facilitate a more rapid and specific diagnosis, which would lead to the implementation of more appropriately targeted antifungal drug therapy.

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