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Rapid Identification of *Candida* Species in Blood Cultures by a Clinically Useful PCR Method

JONG HEE SHIN,[1] FREDERICK S. NOLTE,[2] AND CHRISTINE J. MORRISON[3]*

Department of Clinical Pathology, Chonnam University Medical School, Kwangju, Korea,[1] and Department of Pathology, Emory University Hospital,[2] and Division of Bacterial and Mycotic Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention,[3] Atlanta, Georgia

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Widespread use of fluconazole for the prophylaxis and treatment of candidiasis has led to a reduction in the number of cases of candidemia caused by *Candida albicans* but has also resulted in the emergence of candidemias caused by innately fluconazole-resistant, non-*C. albicans Candida* species. Given the fulminant and rapidly fatal outcome of acute disseminated candidiasis, rapid identification of newly emerging *Candida* species in blood culture is critical for the implementation of appropriately targeted antifungal drug therapy. Therefore, we used a PCR-based assay to rapidly identify *Candida* species from positive blood culture bottles. This assay used fungus-specific, universal primers for DNA amplification and species-specific probes to identify *C. albicans*, *C. krusei*, *C. parapsilosis*, *C. tropicalis*, or *C. glabrata* amplicons. It also used a simpler and more rapid (1.5-h) sample preparation technique than those described previously and used detergent, heat, and mechanical breakage to recover *Candida* species DNA from blood cultures. A simple and rapid (3.5-h) enzyme immunosorbent assay (EIA)-based format was then used for amplicon detection. One hundred fifty blood culture bottles, including 73 positive blood culture bottle sets (aerobic and anaerobic) from 31 patients with candidemia, were tested. The combined PCR and EIA methods (PCR-EIA) correctly identified all *Candida* species in 73 blood culture bottle sets, including bottles containing bacteria coisolated with yeasts and 3 cultures of samples from patients with mixed candidemias originally identified as single-species infections by routine phenotypic identification methods. Species identification time was reduced from a mean of 3.5 days by routine phenotypic methods to 7 h by the PCR-EIA method. No false-positive results were obtained for patients with bacteremias ($n = 18$), artificially produced non-*Candida* fungemias ($n = 3$), or bottles with no growth ($n = 20$). Analytical sensitivity was 1 cell per 2-μl sample. This method is simpler and more rapid than previously described molecular identification methods, can identify all five of the most medically important *Candida* species, and has the potential to be automated for use in the clinical microbiology laboratory.

The incidence of disseminated candidiasis has increased in recent years because of a rise in the number of immunosuppressed and postoperative patients most at risk for the development of this disease (1, 9, 18). The advent of new antifungal drugs has improved the prospects for management of this disease (21); however, diagnosis remains difficult (10, 13, 20). In addition, although fluconazole prophylaxis of bone marrow transplant patients has reduced the incidence of disseminated disease caused by *Candida albicans*, the incidence of primary infections caused by other *Candida* species that are innately resistant to fluconazole, most notably *C. krusei* and *C. glabrata*, has increased (15, 23, 24). Given the rapidly fatal outcome of acute disseminated candidiasis (1, 9), early detection and identification of *Candida* species are critical for the proper targeting of antifungal therapy (15, 19).

Use of PCR-based tests to detect *C. albicans* DNA in body fluids has produced some encouraging results (2, 8, 11). However, routine application of these tests for the detection of candidemia remains challenging. Current methods require labor-intensive sample preparation, costly enzymes for the liberation of *Candida* DNA, and phenol-chloroform extraction to purify DNA before PCR amplification (2, 6). Sensitivity has been variable (2, 6, 14), and false-positive as well as false-negative results have been reported (3).

* Corresponding author. Mailing address: Mailstop G-11, Centers for Disease Control and Prevention, Atlanta, GA 30333. Phone: (404) 639-3128. Fax: (404) 639-3546. E-mail: cjm3@ciddbd2.em.cdc.gov.

On the other hand, routine, culture-based phenotypic identification of *Candida* species from positive blood culture bottles requires at least 1 day following initial positivity to obtain a pure culture, additional time to identify *C. albicans* isolates by germ tube formation, and 2 or more additional days to identify non-*C. albicans* isolates by sugar assimilation strip tests or fermentation tests and cornmeal agar morphology (4). The introduction of CHROMagar *Candida* (Hardy Diagnostics, Santa Monica, Calif.), a differential medium containing chromogenic substrates for the identification of some *Candida* species, can provide rapid, presumptive identification of *C. albicans*, *C. tropicalis*, and *C. krusei* isolates (16); however, additional phenotypic means are still required for confirmation of these species and for the identification of other medically important *Candida* species. Even the 4-h RapID test (Innovative Diagnostics, Norcross, Ga.) requires an additional day or more after initial blood culture positivity to obtain a pure culture from which to inoculate test wells for analysis. Therefore, a test to rapidly and accurately identify *Candida* isolates to the species level, directly from positive blood culture bottles, would be both clinically and epidemiologically useful.

We describe a PCR-based method for the rapid detection and identification of *Candida* isolates from positive blood culture bottles. A simpler extraction method than was previously described for the recovery of *Candida* DNA from blood, employing heat, detergent, and mechanical breakage, was used. No expensive enzymes or toxic phenol-chloroform treatment was required. A simple, rapid, and sensitive microtitration plate enzyme immunosorbent assay (EIA) was then used to detect

PCR products, reducing the time to species identification from a mean of 3.5 days by routine phenotypic identification methods to 7 h by the PCR-EIA method.

MATERIALS AND METHODS

Clinical samples. A total of 150 samples taken from BacT/Alert blood culture bottles (Organon Teknika Corporation, Durham, N.C.), including samples from bottles inoculated with blood from 31 patients with candidemia, were tested. Eight to 10 ml of blood from patients with suspected bacteremia or fungemia was collected at the bedside and was immediately inoculated into each culture bottle (aerobic and anaerobic). Inoculated bottles were incubated at 35 to 37°C for 5 days or until the bottles were positive by colorimetric detection of CO_2. Two-milliliter aliquots from bottles shown to contain yeasts by Gram staining were removed and stored at −30°C. Two-milliliter aliquots from bottles with samples from patients with bacteremia ($n = 18$), bottles with no growth ($n = 20$), or culture bottles artificially seeded with *Blastomyces dermatitidis*, *Aspergillus fumigatus*, or *Histoplasma capsulatum* were also included as negative control samples.

Routine phenotypic culture identification consisted of isolation from positive blood culture bottles on chocolate and Sabouraud dextrose agar plates (BBL, Division of Becton-Dickinson, Cockeysville, Md.), assessment for germ tube and chlamydospore formation, and API 20C (BioMerieux, Hazelwood, Mo.) sugar assimilation strip analysis. Samples giving discrepant results between routine phenotypic and PCR-EIA identification methods were resubcultured on CHROMagar *Candida* plates to identify possible mixed yeast cultures.

To determine the limit of sensitivity of the PCR-EIA, BacT/Alert bottles were first inoculated with 5 ml of whole rabbit's blood, 2-ml aliquots were then removed, and each aliquot was seeded with either 0, 10^1, 10^2, 10^3, 10^4, or 10^5 *C. albicans* (B311) blastoconidia (6) per 200 μl of blood. (Blastoconidia were enumerated with a hemacytometer, and a 200-μl volume was used, to parallel the volume sampled from clinical specimens.) Seeded blood culture bottle fluid was then processed in the same manner as clinical specimens.

Extraction of *Candida* DNA. A mechanical disruption method modified from that of Hopfer et al. (8) was used to extract *Candida* DNA. Two hundred microliters of sample was added to 800 μl of TXTE buffer (10 mM Tris, 1 mM EDTA, 1% Triton X-100 [pH 8.0]) in a sterile, 1.5-ml centrifuge tube, and the mixture was incubated for 10 min at 25°C to lyse the blood cells. Multiple 200-μl samples were tested on different days to confirm the reproducibility of the method. Disrupted blood cell debris was separated from intact *Candida* blastoconidia by centrifugation at 10,000 × *g* for 8 min in a microcentrifuge (Beckman Instruments, Inc., Spinco Division, Palo Alto, Calif.). After three washes by centrifugation with 1 ml of TXTE buffer, blastoconidia were resuspended with 300 μl of TXTE buffer and transferred to a 2-ml, screw-cap, conical-bottom tube (Sarstedt, Inc., Numbrecht, Germany) containing 200 μl of 0.5-mm-diameter zirconium beads (Biospec Products, Bartlesville, Okla.). After boiling in a water bath for 15 min, the mixture was shaken for 20 min in a mechanical cell disrupter (Mini-Beadbeater; Biospec Products). The tubes were then centrifuged at 10,000 × *g* for 20 s, and the supernatant was stored at −20°C until it was used for PCR amplification.

PCR amplification. The fungus-specific, universal primer pair ITS3 (5′-GCA TCG ATG AAG AAC GCA GC-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) was used as described previously (6) to amplify a large portion of the 5.8S ribosomal DNA region, the adjacent ITS2 region, and a small portion of the 28S ribosomal DNA region, generating PCR products of approximately 330 bp for *C. albicans*, 325 bp for *C. tropicalis*, 310 bp for *C. parapsilosis*, 335 bp for *C. krusei*, and 410 bp for *C. glabrata*. The PCR assay was performed with 2 μl of test sample in a total reaction volume of 100 μl, consisting of 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 0.2 mM (each) dATP, dGTP, dCTP, and dTTP, 0.2 μM (each) primer, and 2.5 U of *Taq* DNA polymerase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Thirty cycles of amplification were performed in a model 9600 thermal cycler (Perkin-Elmer Corp., Emeryville, Calif.) after initial denaturation of DNA at 94°C for 5 min. Each cycle consisted of a denaturation step at 95°C for 30 s, an annealing step at 58°C for 30 s, an extension step at 72°C for 1 min, and a final extension at 72°C for 5 min following the last cycle. After amplification, the samples were stored at −20°C until used.

Contamination precautions. Precautions were taken to avoid possible contamination of PCR samples by following the guidelines of Kwok and Higuchi (12) as described by Fujita et al. (6). Appropriate negative controls were included in each test run, including controls omitting either the primer or the DNA template during PCR assays.

Agarose gel electrophoresis. Gel electrophoresis was conducted in TBE buffer (0.1 M Tris, 0.09 M boric acid, 1 mM EDTA [pH 8.4]) at 80 V for 1 to 2 h by using gels composed of 1% (wt/vol) agarose (International Technologies, New Haven, Conn.) and 1% (wt/vol) NuSieve agar (FMC Bioproducts, Rockland, Maine). Gels were stained with 0.5 μg of ethidium bromide (EtBr) per milliliter of distilled H_2O for 10 min, followed by three serial washes for 10 min each with distilled H_2O.

Microtitration plate EIA for the detection of PCR products. Amplicons were detected by using five species-specific probes labeled with digoxigenin and a generic all-*Candida* species probe labeled with biotin in a streptavidin-coated microtiter plate format similar to that described previously (6). Modifications included the use of commercially prepared streptavidin-coated microtitration plates (Boehringer Mannheim), and after the hybridized samples were added, the plates were incubated at ambient temperature for 1 h with shaking by using a microtitration plate shaker (manufactured for Dynatech Industries by CLTI, Middletown, N.Y.). The plates were washed six times with 0.01 M potassium phosphate-buffered saline (pH 7.2) containing 0.05% Tween 20. Each well then received 100 μl of a horseradish peroxidase-conjugated, antidigoxigenin Fab fragment (Boehringer Mannheim) diluted 1:1,000 in hybridization buffer. All remaining steps were performed as described previously (6), except that final color development was allowed to continue for 10 min rather than for 15 min. The absorbance value for the reagent blank, from which DNA was absent but which was replaced with distilled H_2O, was subtracted from the absorbance value for each test sample.

Statistical analysis. Student's *t* test was used to determine differences between sample means. Values are expressed as the mean plus or minus the standard error from the mean. Pearson's *r* was used to calculate the correlation coefficient. *P* values of <0.05 were considered significant.

RESULTS

Sensitivity of DNA detection by PCR-EIA after mechanical breakage of cells. To determine the sensitivity limit of the PCR-EIA by using mechanical breakage of blastoconidia rather than the enzymatic lysis methods described previously (6), whole rabbit's blood was introduced into BacT/Alert culture bottles (ratio of broth to rabbit's blood, 8:1) and 2-ml aliquots were artificially seeded with strain B311 blastoconidia to final concentrations of 0, 10^1, 10^2, 10^3, 10^4, and 10^5 cells per 200 μl. The samples were then treated to mechanically break the blastoconidia and recover the resultant DNA for PCR-EIA as described in the Materials and Methods section. The mean PCR-EIA absorbance values per 200-μl sample from three separate experiments were 0.585 ± 0.053 for 10^5 cells, 0.437 ± 0.055 for 10^4 cells, 0.130 ± 0.030 for 10^3 cells, and 0.024 ± 0.008 for 10^2 cells; no DNA was detected in samples containing 10^1 or no *C. albicans* cells; the mean absorbance at 650 nm for 1 ng of purified DNA amplified by PCR and tested by EIA was 0.547 ± 0.057. The sensitivity of this detection method was therefore 10^2 cells per 200 μl of seeded sample, or 1 cell per 2 μl of sample ($P < 0.025$ versus samples containing no *C. albicans* blastoconidia).

Specificity of probes used for PCR-EIA. DNA probes correctly identified 100% of all single-species cultures for each *Candida* species tested (52 of 52 cultures containing *C. albicans*, 30 of 30 cultures containing *C. glabrata*, 8 of 8 cultures containing *C. parapsilosis*, 7 of 7 cultures containing *C. tropicalis*, and 7 of 7 cultures containing *C. krusei*) without cross-reacting with heterologous *Candida* species DNA (Table 1).

Also, 18 randomly selected samples from patients with bacteremia caused by coagulase-negative staphylococci ($n = 8$), *Enterococcus* spp. ($n = 2$), *Citrobacter freundii* ($n = 2$), *Corynebacterium* sp. strain JK ($n = 2$), *Corynebacterium* sp. (not *C. jekeium*) ($n = 1$), or a mixture of *Enterococcus* spp. and *Staphylococcus aureus* ($n = 2$) or *Klebsiella pneumoniae* and *Acinetobacter calcoaceticus* ($n = 1$) were negative by PCR-EIA (see Bacteremias in Table 1) and demonstrated no detectable PCR amplicon by gel electrophoresis and EtBr staining (data not shown). Twenty inoculated blood culture bottles from patients with suspected sepsis that never became positive during incubation (see No growth in Table 1) were also negative by PCR-EIA. Other negative control samples included BacT/Alert bottles containing cultured *B. dermatitidis*, *A. fumigatus*, or *H. capsulatum* (see Other fungemias in Table 1); all were negative when tested by PCR-EIA with each of the five *Candida* species probes.

Seven *C. albicans*-positive blood culture bottles contained coexisting bacteria identified by routine bacteriological methods as *Enterococcus* spp. ($n = 4$), coagulase-negative staphy-

TABLE 1. Specificity of DNA probes for detection of *Candida* spp. from blood culture bottles by PCR-EIA

Target DNA	Mean ± SE A_{650} with probe for the following:[a]				
	C. albicans	*C. glabrata*	*C. parapsilosis*	*C. tropicalis*	*C. krusei*
C. albicans (n = 52)	0.328 ± 0.026	0[b]	0	0	0
C. glabrata (n = 30)	0	0.702 ± 0.058	0	0	0
C. parapsilosis (n = 8)	0	0	0.602 ± 0.073	0	0
C. tropicalis (n = 7)	0	0	0	0.758 ± 0.189	0
C. krusei (n = 7)	0	0	0	0	0.339 ± 0.045
Bacteremias (n = 18)	0	0	0	0	0
No growth (n = 20)	0	0	0	0	0
Other fungemias (n = 3)	0	0	0	0	0

[a] The mean ± standard error (SE) A_{650} after subtraction of the corresponding reagent blank (no DNA) values.
[b] Means ± standard errors A_{650} after subtraction of reagent blank for all control samples were as follows: with *C. albicans* probe (n = 93), 0 ± 0.0004; with *C. glabrata* probe (n = 115), 0 ± 0.0002; with *C. parapsilosis* probe (n = 137), 0 ± 0.0002; with *C. tropicalis* probe (n = 138), 0 ± 0.0002; and with *C. krusei* probe (n = 138), 0 ± 0.0002.

lococcus (n = 1), or *Acinetobacter lwoffii* (n = 2). All samples were correctly identified by PCR-EIA to contain *C. albicans* without interference from coexisting bacteria. *C. glabrata*, mixed with *Enterobacter aerogenes* (n = 1) in an anaerobic bottle, was also correctly identified as *C. glabrata* by PCR-EIA. PCR amplification of samples containing a single species of *Candida* mixed with bacteria gave a single, *Candida*-specific band upon gel electrophoresis and EtBr staining (data not shown). These results demonstrate that no bacterial DNA was amplified by the fungus-specific primer pair used for PCR and that the PCR-EIA method could accurately detect *Candida* species even in the presence of coexisting bacteria.

Routine phenotypic identification methods versus PCR-EIA to identify *Candida* species. Routine subculture and phenotypic identification of *Candida* species from 31 patients with candidemia demonstrated that of all *Candida* species isolated, 36 were identified as *C. albicans*, 19 were identified as *C. glabrata*, 7 were identified as *C. parapsilosis*, 5 were identified as *C. tropicalis*, 3 were identified as *C. krusei*, and 3 were identified as mixed candidemias caused by two kinds of *Candida* spp. (*C. krusei* plus *C. glabrata* or *C. glabrata* plus *C. albicans*) (Table 2).

PCR-EIA identification results matched phenotypic identification results for 67 of 70 samples (35 *C. albicans*, 17 *C. glabrata*, 7 *C. parapsilosis*, 5 *C. tropicalis*, and 3 *C. krusei*) found to contain a single species by routine phenotypic methods (Table 2). Samples from an aerobic bottle that was found to contain *C. albicans* only by phenotypic methods were identified by PCR-EIA to contain both *C. albicans* and *C. glabrata* (Table 2 and Table 3, patient A). Repeat subculture of the original bottle on CHROMagar *Candida* medium and reidentification by routine phenotypic methods indicated that this specimen contained a mixture of *C. albicans* and *C. glabrata*. Isolation of *C. glabrata*, which comprised only 29.3% of the total colonies recovered from CHROMagar *Candida* medium, was probably obscured during initial routine isolation because of concomitantly heavy growth of coagulase-negative staphylococci (Table 3). Therefore, the PCR-EIA method could detect mixed yeast cultures missed by routine subculturing methods even when blood cultures contained bacteria.

Samples from two aerobic culture bottles inoculated with blood from another patient (Tables 2 and 3, patient B) were found to contain *C. glabrata* alone by routine phenotypic methods but were identified to contain both *C. glabrata* and *C. albicans* by the PCR-EIA method [Table 3, specimens 1B (A) and 3B (A)]. In this case of mixed candidemia, *C. albicans* accounted for only 2.4 to 5.0% of the total cell number recovered on CHROMagar *Candida* plates. Therefore, identification of *C. albicans* may have been missed initially because the percentage of *C. albicans* cells present in the mixture was low. This result indicates that the PCR-EIA is more sensitive than routine phenotypic methods for the detection and identification of small numbers of yeasts in mixed cultures.

Two anaerobic cultures [Tables 2 and 3, patient C, specimens 1C (N) and 2C (N)] were identified by routine phenotypic methods to contain both *C. krusei* and *C. glabrata* but were identified to contain *C. glabrata* only by PCR-EIA and CHROMagar *Candida* medium. However, two counterpart aerobic bottles containing samples taken from the same patient [specimens 1C (A) and 2C (A)] were identified as containing *C. krusei* only by conventional methods, PCR-EIA, and CHROMagar *Candida* subculture (Table 3). This patient was therefore correctly identified as having mixed *C. krusei*-*C. glabrata* candidemia by all three methods when samples from both aerobic and anaerobic bottles were examined.

An aerobic bottle (Tables 2 and 3, patient D) was identified by routine phenotypic methods to contain both *C. glabrata* and *C. albicans* but was identified to contain *C. glabrata* only by PCR-EIA and by CHROMagar *Candida* culture [specimen 1D (A)]. Two other samples received on the same day from the same patient were identified as containing *C. glabrata* only by routine phenotypic methods, PCR-EIA, and CHROMagar *Candida* culture [specimens 2D (A) and 2D (N)]. No detectable growth of *C. albicans* upon resubculture was observed,

TABLE 2. Comparative identification of *Candida* spp. in BacT/Alert blood culture bottle sets by routine phenotypic identification methods versus PCR-EIA

Routine phenotypic identification	No. of bottle sets[a]	PCR-EIA identification	No. of bottle sets[a]
C. albicans	36	*C. albicans*	35
		C. albicans + *C. glabrata*	1
C. glabrata	19	*C. glabrata*	17
		C. glabrata + *C. albicans*	2
C. parapsilosis	7	*C. parapsilosis*	7
C. tropicalis	5	*C. tropicalis*	5
C. krusei	3	*C. krusei*	3
C. krusei + *C. glabrata*	2	*C. krusei* + *C. glabrata*	2
C. glabrata + *C. albicans*	1	*C. glabrata*	1
Total	73		73

[a] A total of 109 blood cultures from 73 culture bottle sets (aerobic and anaerobic) for 31 patients with candidemia.

TABLE 3. Resolution of discrepancies between phenotypic and PCR-EIA methods for the identification of *Candida* species

Patient	Coded specimen no.[a]	Identification by the following method:		
		Routine phenotypic method	PCR-EIA	Final identification[b]
A	1A (A)	*C. albicans* + SCN[c]	*C. albicans* + *C. glabrata*	*C. albicans* + *C. glabrata*
B	1B (A)	*C. glabrata*	*C. glabrata* + *C. albicans*	*C. glabrata* + *C. albicans*
	1B (N)	*C. glabrata*	*C. glabrata*	*C. glabrata*
	2B (A)	*C. glabrata*	*C. glabrata*	*C. glabrata*
	2B (N)	*C. glabrata*	*C. glabrata*	*C. glabrata*
	3B (A)	*C. glabrata*	*C. glabrata* + *C. albicans*	*C. glabrata* + *C. albicans*
	3B (N)	*C. glabrata*	*C. glabrata*	*C. glabrata*
C	1C (A)	*C. krusei*	*C. krusei*	*C. krusei*
	1C (N)	*C. krusei* + *C. glabrata*	*C. glabrata*	*C. glabrata*
	2C (A)	*C. krusei*	*C. krusei*	*C. krusei*
	2C (N)	*C. krusei* + *C. glabrata*	*C. glabrata*	*C. glabrata*
D	1D (A)	*C. glabrata* + *C. albicans*	*C. glabrata*	*C. glabrata*
	2D (A)	*C. glabrata*	*C. glabrata*	*C. glabrata*
	2D (N)	*C. glabrata*	*C. glabrata*	*C. glabrata*

[a] (A), aerobic culture bottle; (N), anerobic culture bottle.
[b] Final species identification was corroborated by colony morphology and color on CHROMagar *Candida* medium and by reidentification by routine phenotypic methods.
[c] *C. glabrata* identification during initial subculture was obscured by overgrowth with coagulase-negative staphylococci (SCN).

indicating that *C. albicans* was a laboratory contaminant of the initial subculture procedure and that this patient had candidemia due to *C. glabrata* only (Tables 2 and 3). Therefore, the PCR-EIA method correctly identified all cases of candidemia in 73 clinical blood culture bottle sets including 70 cultures of blood from patients with single-species candidemia and 3 cultures of blood from patients with mixed candidemia (identified to contain a single *Candida* species by routine phenotypic methods).

The mean time from the first positive blood culture bottle to species identification by routine phenotypic methods (subculture, germ tube and chlamydospore formation tests, and API 20C) was 3.5 days (84 h). More slowly growing, individual isolates took as long as 15.5 days for growth and species identification by routine phenotypic methods (379.2 h, *C. parapsilosis* isolate). For the PCR-EIA, on the other hand, only 1.5 h was required to disrupt and isolate DNA from positive blood culture bottles, 2 h was required for PCR amplification of isolated DNA, and 3.5 h was required to perform EIA detection of the PCR product. Therefore, once the bottles became positive, species could be identified in as little as 7 h by the PCR-EIA method, including the time required to prepare PCR and EIA reagent mixtures, in contrast to routine phenotypic methods which took several days.

Number of *Candida* cells recovered from BacT/Alert blood culture bottles and its relationship to mean EIA absorbance values. To determine if the mean EIA absorbance value could be used to roughly quantitate the number of cells present in each blood culture bottle, cells in each bottle were enumerated with a hemacytometer, and the resultant number was compared to the EIA absorbance values obtained for these specimens. Of the 104 samples containing a single *Candida* species, 29 (28%) contained more than 10^6 *Candida* cells, 50 (48%) contained between 10^5 and 10^6 cells, 18 (17%) contained 10^4 to

TABLE 4. Number of *Candida* cells in positive BacT/Alert blood culture bottle aliquots containing a single species and its relationship to mean PCR-EIA absorbance value

No. of cells per 200-μl sample[a]	Mean ± SE A_{650} by EIA for designated *Candida* species[b]					
	All species	*C. albicans*	*C. glabrata*	*C. parapsilosis*	*C. krusei*	*C. tropicalis*
$>10^6$	0.550 ± 0.055 (29)	0.500 ± 0.090 (11)	0.640 ± 0.111 (10)	0.950 (1)	0.370 ± 0.031 (5)	0.820 ± 0.121 (2)
10^5	0.550 ± 0.047 (50)	0.350 ± 0.039 (21)	0.770 ± 0.068 (16)	0.550 ± 0.060 (7)	0.260 ± 0.156 (2)	0.900 ± 0.280 (4)
10^4	0.350 ± 0.057 (18)	0.270 ± 0.038 (16)	0.930 ± 0.071 (2)			
10^3	0.190 ± 0.040 (5)	0.230 ± 0.053 (3)	0.180 (1)			0.080 (1)
$<10^3$	0.140 ± 0.071 (2)	0.070 (1)	0.220 (1)			
Total	0.498 ± 0.030 (104)	0.328 ± 0.026 (52)	0.702 ± 0.058 (30)	0.602 ± 0.073 (8)	0.339 ± 0.045 (7)	0.758 ± 0.189 (7)

[a] The numbers of *Candida* cells present are expressed per 200-μl sample because this was the sample volume used for each PCR.
[b] The number of bottles containing the given cell number are indicated in parentheses.

10^5 cells, 5 (5%) contained 10^3 to 10^4 cells, and 2 (2%) contained fewer than 10^3 cells per 200 μl (Table 4). Taken together, of all 109 samples from patients with candidemia tested, including those from patients with mixed candidemia, 82 (75.2%) contained more than 10^5 *Candida* cells, 20 samples (18.3%) contained between 10^4 and 10^5 cells, 5 (4.6%) contained between 10^3 and 10^4 cells, and 2 samples (1.8%) contained fewer than 10^3 cells per 200 μl. When analyzed by individual species, the greatest number of culture bottles contained 10^5 cells except for *C. krusei*, for which more bottles contained 10^6 cells or more (Table 4).

The mean PCR-EIA absorbance value for all species taken together was proportional to the number of cells present (Pearson's $r = 0.97$) but plateaued at 10^5 cells or greater (0.550 ± 0.055 for more than 10^6 cells and 0.550 ± 0.047 for 10^5 cells; see data for all species in Table 4).

The mean A_{650} values obtained for samples seeded with known numbers of blastoconidia (described in the first paragraph of the Results section) were very similar to those obtained in practice for a comparable number of cells recovered from single-species cultures of BacT/Alert blood culture bottles (Table 4). These results indicate that since as few as 10^2 cells per 200 μl could be detected by PCR-EIA of seeded samples, the detection limit of this method is well within the sensitivity needed to detect 100% of all positive blood culture bottles tested in practice.

DISCUSSION

The recent increase in infections caused by non-*C. albicans Candida* species (15, 23, 24) and the innate resistance of some species to certain antifungal drugs (15, 19, 21) make a test for rapidly differentiating *Candida* species valuable for targeted drug therapy. We therefore developed a PCR-based assay to identify *Candida* species directly from positive blood culture bottles without further subculturing. This is the first clinical application of universal, fungus-specific primers and species-specific probes used in the format described previously (6). The advantages of this method include (i) a simpler DNA extraction method than those described previously for the recovery of *Candida* DNA from blood cultures, (ii) no required use of toxic (phenol-chloroform) or expensive (proteinase K and Zymolyase) chemicals, (iii) a nonisotopic EIA format for the detection of PCR products, (iv) a total time from blood culture bottle positivity to species identification of 7 h versus a mean of 3.5 days by routine phenotypic culture identification methods, (v) identification of more than one species of yeast in mixed cultures with no cross-reactivity with bacteria, other fungi, or other yeast species, and (vi) no interference with the detection of yeasts in blood cultures containing bacteria (in contrast to routine phenotypic identification methods, in which *C. glabrata* was not detected in one culture due to overgrowth by coagulase-negative staphylococci). The last item listed is an especially valuable feature of the PCR-EIA method because mixed yeast and bacterial blood cultures can frequently occur in the immunosuppressed patient population most at risk for disseminated candidiasis due to the increased use of indwelling catheters in these patients (1, 5, 9). An additional advantage of the PCR-EIA method is that it is easily amenable to automation, making this test particularly attractive for use in the clinical microbiology laboratory setting. Given the rapidly fatal course of acute, disseminated candidiasis (1, 9, 20), the capacity to more rapidly and more accurately diagnose candidemia could result in significant savings and benefits to hospitals and patients in lives lost, in inappropriate or unnecessary drug administration, and in hospitalization time.

Only five species of *Candida* were isolated from blood cultures at Emory University Hospital during the study period (listed in order of decreasing incidence): *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, and *C. krusei*. These five species are also the most common isolates found in cultures of blood samples at other tertiary care hospitals (5); however, because the spectrum of *Candida* species causing candidemia may vary in different hospitals, probes could be tailored to detect the most common species found in a given hospital. Work is under way in the Centers for Disease Control and Prevention's Mycotic Diseases Laboratories to expand the menu of probes to include all of the medically important species of *Candida* as well as the other common opportunistic and systemic fungal pathogens.

We chose to use positive blood culture bottles as a practical alternative to whole-blood specimens for our initial evaluation of the PCR-EIA method. Previously described PCR methods for the direct detection of *Candida* DNA in blood used cumbersome and time-consuming sample preparation methods and still lacked the desired sensitivity (7, 14) to diagnose up to one-half of all candidemias (10, 20). A major limitation of PCR-based methods for the direct detection of *Candida* in blood is the small effective sample volume used, typically 0.1 to 1.0 ml of whole blood (7, 14, 22). In contrast, blood culture bottles are generally inoculated with 8 to 10 ml of blood. It is unlikely that the sensitivities of PCR-based methods that use whole blood as the target specimen will approach the sensitivity of blood culture for the direct detection of candidemia until these methods can accommodate larger effective sample volumes. Also, the growth of *Candida* cells in bottles during culture of blood increases the concentration of target DNA available for extraction and PCR amplification. The 1:5 dilution of patient blood with blood culture medium may also effectively dilute out PCR-inhibitory factors commonly found in whole blood (7, 17), making simple DNA extraction methods feasible without the need for additional cumbersome purification steps. Further decreases in the time needed for detection and identification may be achieved by testing aliquots of blood culture bottles early, prior to detection of growth by the blood culture instrument.

Using this simpler sample preparation method and our previously described PCR-EIA detection method (6), the analytical sensitivity of the test was 10^2 cells/200 μl or 1 cell/2-μl sample, a log higher than that for detection by gel electrophoresis and EtBr staining (data not shown). The analytical sensitivity of the PCR-EIA was more than sufficient for the application described here because only two samples (1.8%) contained fewer than 10^3 cells/200 μl and the *Candida* species in both of these samples were detected and correctly identified by PCR-EIA. This indicates that as soon as BacT/Alert blood culture bottles become detectably positive, the PCR-EIA could be run effectively.

Enzymatic treatment of *Candida* cells, followed by extensive purification of DNA from whole blood by methods previously described by our laboratory, increased the analytical sensitivity of the PCR-EIA by approximately 1 log (6). Therefore, enzymatic treatment (6, 20, 22) may be necessary when whole blood rather than blood cultures are analyzed because lower cell numbers are present and PCR-inhibitory factors are not diluted out (7, 17). Future efforts will be directed toward the development of a simple sample preparation method with performance characteristics acceptable for the direct detection of *Candida* species from whole blood.

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REFERENCES

1. **Beck-Sagué, C., and W. R. Jarvis.** 1993. Secular trends in the epidemiology of nosocomial fungal infections in the United States, 1980–1990. National Nosocomial Infections Surveillance System. J. Infect. Dis. **167:**1247–1251.
2. **Buchman, T. G., M. Rossier, W. G. Merz, and P. Charache.** 1990. Detection of surgical pathogens by in vitro DNA amplification. Part I. Rapid identification of *C. albicans* by in vitro amplification of a fungus-specific gene. Surgery **108:**338–347.
3. **Burgener-Kairuz, P., J.-P. Zuber, P. Jaunin, T. G. Buchman, J. Billie, and M. Rossier.** 1994. Rapid detection and identification of *Candida albicans* and *Torulopsis* (*Candida*) *glabrata* in clinical specimens by species-specific nested PCR amplification of a cytochrome P-450 lanosterol-α-demethylase (L1A1) gene fragment. J. Clin. Microbiol. **32:**1902–1907.
4. **Dooley, D. P., M. L. Beckius, and B. S. Jeffrey.** 1994. Misidentification of clinical yeast isolates by using the updated Vitek Yeast Biochemical Card. J. Clin. Microbiol. **32:**2889–2892.
5. **Fraser, V. J., M. Jones, J. Dunkel, S. Storfer, G. Medoff, and W. C. Dunagan.** 1992. Candidemia in a tertiary care hospital: epidemiology, risk factors, and predictors of mortality. Clin. Infect. Dis. **15:**414–421.
6. **Fujita, S.-I., B. A. Lasker, T. J. Lott, E. Reiss, and C. J. Morrison.** 1995. Microtitration plate enzyme immunoassay to detect PCR-amplified DNA from *Candida* species in blood. J. Clin. Microbiol. **33:**962–967.
7. **Holmes, A. R., Y. C. Lee, R. D. Cannon, H. F. Jenkinson, and M. G. Shepard.** 1992. Yeast-specific DNA probes and their application for the detection of *Candida albicans*. J. Med. Microbiol. **37:**346–351.
8. **Hopfer, R. L., P. Walden, S. Setterquist, and W. E. Highsmith.** 1993. Detection and differentiation of fungi in clinical specimens using polymerase chain reaction (PCR) amplification and restriction enzyme analysis. J. Med. Vet. Mycol. **31:**65–75.
9. **Jarvis, W. R.** 1995. Epidemiology of nosocomial fungal infections, with emphasis on *Candida* species. Clin. Infect. Dis. **20:**1526–1530.
10. **Jones, J. M.** 1990. Laboratory diagnosis of invasive candidiasis. Clin. Microbiol. Rev. **3:**32–45.
11. **Kan, V. L.** 1993. Polymerase chain reaction for the diagnosis of candidemia. J. Infect. Dis. **168:**799–783.
12. **Kwok, S., and R. Higuchi.** 1989. Avoiding false positives with PCR. Nature (London) **339:**237–238.
13. **Matthews, R. C.** 1993. Early diagnosis of systemic *Candida* infection. J. Antimicrob. Chemother. **31:**809–812.
14. **Miyakawa, Y., T. Mabuchi, and Y. Fukazawa.** 1993. New method for detection of *Candida albicans* in human blood by polymerase chain reaction. J. Clin. Microbiol. **31:**3344–3347.
15. **Nguyen, M. H., J. E. Peacock, Jr., A. J. Morris, D. C. Tanner, M. L. Nguyen, D. R. Snydman, M. M. Wagener, M. G. Rinaldi, and V. L. Yu.** 1996. The changing face of candidemia: emergence of non-*Candida* species and antifungal resistance. Am. J. Med. **100:**617–623.
16. **Odds, F. C., and R. Bernaerts.** 1994. CHROMagar *Candida*, a new differential isolation medium for presumptive identification of clinically important *Candida* species. J. Clin. Microbiol. **32:**1923–1929.
17. **Panaccio, M., and A. Lew.** 1991. PCR based diagnosis in the presence of 8% (v/v) blood. Nucleic Acids Res. **19:**1151.
18. **Pfaller, M. A.** 1995. Epidemiology of fungal infections. J. Hosp. Infect. **30**(Suppl)**:**329–338.
19. **Price, M. F., M. T. LaRocco, and L. O. Gentry.** 1994. Fluconazole susceptibilities of *Candida* species and distribution of species recovered from blood cultures over a 5-year period. Antimicrob. Agents Chemother. **38:**1422–1424.
20. **Reiss, E., and C. J. Morrison.** 1993. Nonculture methods for diagnosis of disseminated candidiasis. Clin. Microbiol. Rev. **6:**311–323.
21. **Rex, J. H., M. A. Pfaller, A. L. Barry, P. W. Nelson, and C. D. Webb for the NIAID Mycoses Study Group and the Candidemia Study Group.** 1994. Antifungal susceptibility testing of isolates from a randomized, multicenter trial of fluconazole versus amphotericin B for the treatment of nonneutropenic patients with candidemia. Antimicrob. Agents Chemother. **38:**1422–1424.
22. **van Deventer, A. J. M., W. H. F. Goessens, A. van Belkum, H. J. A. van Vliet, E. W. M. van Etten, and H. A. Verbrugh.** 1995. Improved detection of *Candida albicans* by PCR in blood of neutropenic mice with systemic candidiasis. J. Clin. Microbiol. **33:**625–628.
23. **Wingard, J. R.** 1995. Importance of *Candida* species other than *C. albicans* as pathogens in oncology patients. Clin. Infect. Dis. **20:**115–125.
24. **Wingard, J. R., W. G. Merz, M. G. Rinaldi, T. R. Johnson, J. E. Karp, and R. Saral.** 1991. Increase in *Candida krusei* infections among patients with bone marrow transplantation and neutropenia treated prophylactically with fluconazole. N. Engl. J. Med. **325:**1274–1277.