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Microtitration Plate Enzyme Immunoassay To Detect PCR-Amplified DNA from *Candida* Species in Blood

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We developed a microtitration plate enzyme immunoassay to detect PCR-amplified DNA from *Candida* species. Nucleotide sequences derived from the internal transcribed spacer (ITS) region of fungal rDNA were used to develop species-specific oligonucleotide probes for *Candida albicans*, *C. tropicalis*, *C. parapsilosis*, and *C. krusei*. No cross-hybridization was detected with any other fungal, bacterial, or human DNAs tested. In contrast, a *C.* (*Torulopsis*) *glabrata* probe cross-reacted with *Saccharomyces cerevisiae* DNA but with no other DNAs tested. Genomic DNA purified from *C. albicans* blastoconidia suspended in blood was amplified by PCR with fungus-specific universal primers ITS3 and ITS4. With the *C. albicans*-specific probe labeled with digoxigenin, a biotinylated capture probe, and streptavidin-coated microtitration plates, amplified DNA from as few as two *C. albicans* cells per 0.2 ml of blood could be detected by enzyme immunoassay.

Disseminated candidiasis is an important infectious complication in patients who have undergone cardiac or abdominal surgery or in patients who are severely granulocytopenic as a result of therapies for bone marrow transplantation or cancer (16, 27, 29). Antemortem diagnosis of disseminated candidiasis is difficult because the clinical presentation is usually nonspecific and antibody production in immunocompromised patients can be variable, complicating the diagnosis (14). Although two or more positive blood cultures are often used to identify disseminated disease, standard blood culturing methods can require 2 to 5 days for detection and even longer for species identification (14). To shorten the time required to obtain an accurate diagnosis independent of a functioning immune system, laboratory tests have been developed to detect circulating *Candida* cell wall mannan (8, 21), enolase (35), or D-arabinitol (8, 14) for rapid diagnosis of disseminated candidiasis. However, the sensitivity of these tests varies among investigators and is reported to range from 22 to 100% (14).

The development of DNA-based methods for detection of *Candida* spp. provides an alternative and potentially more sensitive means to diagnose disseminated candidiasis. Southern blotting of nonamplified DNA targets has a detection limit of approximately 500 to 10^5 blastoconidia, depending on the method and probe used (6, 11). PCR technology (30) was recently adapted to amplify *Candida albicans* DNA, facilitating its detection (3, 7, 10, 25). However, detection of *C. albicans* DNA recovered from clinical specimens, even after PCR amplification, lacks sensitivity and is cumbersome for most laboratories (3, 7), particularly when DNA is recovered from blood (10, 24). Sensitivity can be improved to 10 cells per ml (15) or 3 cells per 0.1 ml (24), but this requires the use of Southern blotting coupled with radioisotopically labeled probes for detection.

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In the present study, *Candida* sp. 5.8S rRNA genes and the adjacent internal transcribed spacer (ITS) regions were PCR amplified by using fungus-specific universal primers ITS3 and ITS4 (36). Nonisotopic, digoxigenin-labeled oligonucleotide probes were designed on the basis of the sequence of the ITS2 region of *C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. krusei*, and *C.* (*Torulopsis*) *glabrata* rDNA (17). These probes were then used in a microtitration plate enzyme immunoassay (EIA) to rapidly detect and identify amplified genomic DNA from *C. albicans* blastoconidia introduced into blood.

MATERIALS AND METHODS

Microorganisms and reagents. *C. albicans* B311 and H317 and *Saccharomyces cerevisiae* AB972 were obtained as previously described (19). *C. tropicalis* WO745, *C. parapsilosis* WO471, *C. guilliermondii* WO411, *C. krusei* WO701, *C.* (*Torulopsis*) *glabrata* WO756, and *Cryptococcus neoformans* var. *neoformans* 90-6 were obtained from lyophilized stock cultures maintained in the Mycology Culture Collection, Emerging Bacterial and Mycotic Diseases Branch, Centers for Disease Control and Prevention (CDC). The identity of yeast isolates was determined by carbohydrate assimilation tests performed with the API20C kit (bioMerieux Vitek, Inc., Hazelwood, Mo.), germ tube formation in serum, morphology on cornmeal agar, or for *C. neoformans* identification, pigment production on DL-3,4-dihydroxyphenylalanine agar. Filamentous fungal isolates *Aspergillus fumigatus* 91-019720, *A. flavus* 91-019724, *Histoplasma capsulatum* G217B, *Penicillium marneffei* B-3420, and *Blastomyces dermatitidis* 4478 were obtained from the Mycology Culture Collection, Emerging Bacterial and Mycotic Diseases Branch, CDC. Filamentous fungi were identified by colonial and microscopic morphology. *Escherichia coli* DH5α was obtained from Bethesda Research Laboratories (Gaithersburg, Md.). *Staphylococcus aureus* ATCC 1126 DNA was kindly provided by Tammy Bannerman (Hospital Infections Program, CDC), and *Pseudomonas aeruginosa* ATCC 10332 DNA was kindly provided by Arnold Steigerwalt (Division of Bacterial and Mycotic Diseases, CDC). Genomic DNA from human placenta was kindly provided by David Swan (Division of Viral and Rickettsial Diseases, CDC). All chemicals were purchased from Sigma Chemical Co., St. Louis, Mo., unless otherwise specified.

Precautions against contamination. Universal precautions suggested by Kwok and Higuchi (18) were used to eliminate possible contamination of samples. Cross-contamination by aerosols was reduced by physical separation of laboratory areas used to prepare PCRs and to analyze PCR products and by using a combination of positive-displacement pipetters and aerosol-resistant pipette tips. Other precautions included autoclaving of buffers and distilled water used for PCRs, use of fresh lots of previously aliquoted PCR reagents, and testing of appropriate negative controls, including omission of either the primer or the DNA template during the PCR.

Purification of target DNA. *Candida* species and *S. cerevisiae* isolates were grown in 10 ml of YPG broth (1% yeast extract, 2% Bacto Peptone, 1% glucose; Difco Laboratories, Detroit, Mich.) at 37°C, and DNA was purified from lysed spheroplasts as described by Lasker et al. (20). *C. neoformans* genomic DNA was purified from lysed spheroplasts as described by Restrepo and Barbour (28).

Filamentous fungi were grown in 50 ml of YPG broth at 30°C for 48 h. Mycelia were harvested by filtration, washed once with sterile distilled water, and ground with a mortar and pestle in the presence of liquid nitrogen in a laminar flow biological safety cabinet. Genomic DNA was then isolated as described by Spitzer et al. (33) by repeated phenol-chloroform and chloroform extractions. Bacterial DNA was isolated by standard methods (23).

Preparation of template DNA from *C. albicans* blastoconidia suspended in blood. Blood (10 ml) from New Zealand White rabbits (Myrtle's Rabbitry, Memphis, Tenn.) was collected from a central ear artery into lysis-centrifugation tubes (Wampole Laboratories, Cranbury, N.J.) in accordance with CDC Animal Care and Use Committee guidelines. Human blood was also tested in preliminary experiments with similar results.

To determine the sensitivity of the prototype test, blood was seeded with known numbers of *Candida* blastoconidia. Knowing the exact number of (viable plus nonviable) cells present was important for determining test sensitivity, since PCR methods can detect DNA from dead, as well as viable, blastoconidia (unpublished observation). Therefore, 0, 10^1, 10^2, 10^3, or 10^4 *C. albicans* B311 blastoconidia which had been enumerated microscopically with a hemacytometer were seeded into 1 ml of collected blood. Erythrocytes and leukocytes were lysed by adding 0.8 ml of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) containing 0.05% (wt/vol) proteinase K (15 U/mg) and 0.05% (vol/vol) Tween 20 detergent to 0.2 ml of seeded blood. After incubation at 55°C for 30 min, blastoconidia were centrifuged at 10,000 × *g* for 8 min at 20°C in a Beckman Microfuge and washed twice with TE buffer containing 0.5% Tween 20 and twice with SE solution (1 M sorbitol–0.1 M EDTA, pH 7.5).

Spheroplasts were prepared by adding 0.5 ml of SE solution containing 0.1% (wt/vol) Zymolyase-100T (100,000 U/g; Seikagaku Corp., Tokyo, Japan) and 1% (vol/vol) 2-mercaptoethanol to the cell pellets. After incubation at 37°C for 30 min and an additional 30 min of incubation at 25°C on a rocker platform (20 cycles per min), spheroplasts were centrifuged at 7,000 × *g* for 5 min at 20°C and washed twice with SE solution. Spheroplasts were lysed in 0.4 ml of TE buffer containing 0.05% (wt/vol) proteinase K (15 U/mg) and 0.5% (vol/vol) Tween 20, incubated at 55°C for 1 h, and then heated at 95°C for 10 min to inactivate the proteinase K. Nucleic acids were extracted by adding an equal volume of TE-saturated phenol-chloroform (1 volume of TE-saturated phenol to 1 volume of chloroform) and vortex mixing for 1 min. The emulsion was centrifuged at 10,000 × *g* for 5 min, and the aqueous phase was extracted by adding an equal volume of chloroform. One-tenth volume of 3.0 M sodium acetate buffer (pH 5.2) was then added to the resultant aqueous phase, and DNA was precipitated by adding 2 volumes of cold isopropanol and placing the samples at −20°C for 1 h. Precipitated nucleic acids were then collected by centrifugation at 10,000 × *g* for 10 min at 4°C. One milliliter of 70% ice-cold ethanol was added to wash the pellet, and samples were centrifuged for an additional 5 min at 10,000 × *g*. Nucleic acids were then vacuum dried and resuspended in 30 μl of distilled water or TE buffer.

Oligonucleotide synthesis of primers and probes. Synthetic oligodeoxyribonucleotides were prepared by β-cyanoethyl phosphoramidite chemistry with a 380B automated DNA synthesizer (Applied Biosystems, Foster City, Calif.). The oligonucleotide primer pair ITS3 and ITS4 was previously shown to amplify fungal 5.8S rDNA and the adjacent ITS region (36). Oligonucleotide probes specific for *Candida* species were prepared from sequences of the rDNA ITS2 region (17, 22, 36). Oligonucleotide probes CA, CT, CP, CK, and CG were designed to detect *C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. krusei*, and *C.* (*Torulopsis*) *glabrata*, respectively. For probes CA, CT, and CP, nonhomologous 3′ regions of ITS2 were derived from GenBank entries L07796, L11349, and L11352, respectively. Probes CG and CK were developed on the basis of data previously reported (17).

Oligonucleotide probes were initially synthesized with a 5′-terminal amine group (Aminolink 2; ABI, Foster City, Calif.). Amino-linked oligonucleotides were mixed with a 10-fold molar excess of digoxigenin–3-*O*-methylcarbonyl-ε-aminocaproic acid *N*-hydroxysuccinimide ester (Boehringer Mannheim, Indianapolis, Ind.) in 0.1 M sodium carbonate buffer, pH 9.0. After overnight incubation at ambient temperature, the digoxigenin-conjugated oligonucleotides were purified by reverse-phase high-pressure liquid chromatography (2).

The 5.8S rDNA consensus oligonucleotide probe BP was labeled with biotin by incorporating dimethoxytrityl-biotin-carbon 6-phosphoramidite (Cambridge Research Biochemicals, Inc., Wilmington, Del.) at the 5′ end during standard synthesis on a 380B DNA synthesizer. The dimethoxytrityl group was retained on the biotinylated oligonucleotide to facilitate purification by reverse-phase high-pressure liquid chromatography (2). ITS4P (5′-end-protected 3′ primer) was synthesized as previously described (34).

PCR conditions. A Gene Amp DNA amplification reagent kit (Perkin-Elmer Corp., Norwalk, Conn.) was used for PCR amplification of genomic DNA. The reaction mixture (50 μl) contained 50 mM KCl; 1.5 mM $MgCl_2$; 0.001% gelatin; 10 mM Tris-HCl (pH 8.3); 50 μM each dATP, dCTP, dGTP, and dTTP; 0.1 μM each primer; 5 μl of template DNA, and 1.25 U of *Taq* DNA polymerase. A primer concentration of 0.1 μM was found by titration to be optimal for DNA amplification with minimal primer dimerization (unpublished observations). Samples were overlaid with 30 μl of mineral oil prior to PCR amplification in a Perkin Elmer Cetus DNA thermal cycler. *Taq* polymerase was added after the thermal cycler reached 94°C and before initiation of temperature cycling. PCR amplification was determined to be optimum after 30 cycles of denaturation at 95°C for 1 min, primer annealing at 55°C for 1 min, and extension at 72°C for 1.5 min. After the last cycle, final DNA extension was performed at 72°C for 5 min.

Agarose gel electrophoresis. Electrophoresis was conducted in TBE buffer (0.089 M Tris, 0.089 M boric acid, 0.02 M EDTA, pH 8.4) at 80 V for 1 to 2 h on gels composed of 1% (wt/vol) agarose (International Technologies, Inc. New Haven, Conn.) and 1% (wt/vol) NuSieve (FMC Bioproducts, Rockland, Maine).

Microtitration plate hybridization assay. PCR-amplified DNA was hybridized to digoxigenin- and biotin-labeled oligonucleotide probes and detected in an EIA by capture with streptavidin-coated microtitration plates (Immulon 2; Dynatech Laboratories, Inc., Chantilly, Va.) (9). To facilitate hybridization, single-stranded DNA was prepared from double-stranded PCR products by either exonuclease digestion of the non-phosphothioate-protected strand (9) or heat denaturation as described below.

Ten microliters of the PCR product was supplemented with dithiothreitol to 1 mM and digested with 0.4 U of T7 gene 6 exonuclease (United States Biochemical, Cleveland, Ohio) per μl for 15 min at 37°C. The digested product was then heated at 75°C for 15 min to inactivate the exonuclease. Alternatively, 10 μl of the PCR product was heated at 95°C for 5 min and then immediately cooled on ice. The single-stranded PCR product obtained by either exonuclease digestion or heating was added to 0.2 ml of hybridization solution (4× SSC [saline sodium citrate buffer; 0.6 M NaCl, 0.06 M trisodium citrate, pH 7.0] containing 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES], 2 mM EDTA, and 0.15% [vol/vol] Tween 20) supplemented with 50 ng each of biotin- and digoxigenin-labeled probes per ml.

Hybridization reactions were performed in 0.5-ml Eppendorf tubes at 37°C for 1 h. After hybridization, 100 μl of each sample was added to duplicate wells of the streptavidin-coated microtitration plate (9), and the plate was incubated at ambient temperature for 1 h with shaking (Minishaker, manufactured for Dynatech by CLTI, Middletown, N.Y.). After washing with potassium phosphate-buffered saline containing 0.05% Tween 20, 100 μl of peroxidase-conjugated, anti-digoxigenin Fab fragment (Boehringer Mannheim) diluted 1:2,000 in hybridization buffer was added per well. Plates were subsequently washed six times with potassium phosphate-buffered saline–0.05% Tween 20. Each well received 100 μl of a mixture of 1 volume of 3, 3′, 5, 5′-tetramethylbenzidine peroxidase substrate (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) and 1 volume of peroxidase solution (Kirkegaard & Perry). The plates were then placed at ambient temperature for 15 min, and the A_{650} of each well was determined with a microtitration plate reader (UV Max; Molecular Devices, Inc., Menlo Park, Calif.). The absorbance of a reagent blank, in which the test sample was replaced with distilled water, was subtracted from each test sample.

Statistical analyses. Student's *t* test was used to determine significant differences between means plus or minus the standard deviation from the mean. *P* values of <0.05 were considered significant.

RESULTS

PCR amplification of fungal rDNA with universal fungal primers ITS3 and ITS4. All of the oligodeoxyribonucleotide primers and oligonucleotide probes used in this study are described in Table 1. One nanogram of genomic DNA from each isolate was amplified by PCR with the universal fungal primer pair ITS3 and ITS4. Use of this primer pair resulted in amplification of DNAs from all of the fungi examined, including *C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. guilliermondii*, *C. krusei*, *C.* (*Torulopsis*) *glabrata*, *S. cerevisiae*, *C. neoformans*, *A. fumigatus*, *A. flavus*, *P. marneffei*, *B. dermatitidis*, and *H. capsulatum* (Fig. 1). No amplicon was detected with DNA isolated from *S. aureus*, *E. coli*, *P. aeruginosa*, or a human placental cell line. The following amplicon sizes were obtained for *Candida* species: 330 bp for *C. albicans*, 325 bp for *C. tropicalis*, 310 bp for *C. parapsilosis*, 260 bp for *C. guilliermondii*, 335 bp for *C. krusei*, and 410 bp for *C.* (*Torulopsis*) *glabrata* (Fig. 1). Fungi tested not belonging to a *Candida* species yielded amplicons with sizes ranging from 340 to 410 bp (Fig. 1).

Comparison of EIA sensitivity for detection of a PCR product by heat denaturation versus exonuclease digestion. Two methods for the production of single-stranded capture DNA to coat microtitration plates were compared. One method used heat to denature DNA into single strands, whereas the other

TABLE 1. Synthetic oligonucleotides used in PCR and hybridization analyses

Primer or probe	Nucleotide sequence (5′ to 3′)	Chemistry and location
ITS3	GCA TCG ATG AAG AAC GCA GC	5.8S rDNA universal 5′ primer
ITS4	TCC TCC GCT TAT TGA TAT GC	28S rDNA universal 3′ primer
ITS4P	T[a]C[a]C[a] TCC GCT TAT TGA TAT GC	Phosphorothioate-protected 28S rDNA universal 3′ primer
CA	AT TGC TTG CGG CGG TAA CGT CC	5′-end-labeled digoxigenin probe; ITS region of *C. albicans*
CT	AA CGC TTA TTT TGC TAG TGG CC	5′-end-labeled digoxigenin probe; ITS region of *C. tropicalis*
CP	AC AAA CTC CAA AAC TTC TTC CA	5′-end-labeled digoxigenin probe; ITS region of *C. parapsilosis*
CK	GG CCC GAG CGA ACT AGA CTT TT	5′-end-labeled digoxigenin probe; ITS region of *C. krusei*
CG	TA GGT TTT ACC AAC TCG GTG TT	5′-end-labeled digoxigenin probe; ITS region of *C.* (*Torulopsis*) *glabrata*
BP	CA TGC CTG TTT GAG CGT C(GA)T T	5′-end-labeled, biotinylated probe; 5.8S rDNA

[a] Phosphorothioate protected.

method used T7 gene 6 exonuclease to selectively digest one non-phosphorothioate-protected strand of double-stranded DNA (9). No significant difference in EIA sensitivity was noted when microtitration plates were coated with single-stranded DNA produced by either of these methods (data not shown). Since heat denaturation was more rapid and simpler to perform than exonuclease digestion, all of the results reported here used heat denaturation of DNA before coating of microtitration plates.

Specificity of digoxigenin-labeled probes. Oligonucleotide probes used to detect a specific *Candida* species were designed from sequences of the ITS2 region of rDNA located between the 5.8S and 26S rRNA genes (17, 22; Table 1). Absorbance values for the reaction of the digoxigenin-labeled probes with PCR-amplified DNAs from *Candida* spp. and from control microorganisms in the microtitration plate EIA are shown in Table 2. The probes used to detect *C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. krusei*, and *C.* (*Torulopsis*) *glabrata* were designated CA, CT, CP, CK, and CG, respectively (Table 2). Reaction of probe CA with PCR-amplified *C. albicans* DNA resulted in a mean A_{650} of 0.564 (Table 2). This is in contrast to the lack of reactivity observed for probe CA when DNAs from other fungi, other *Candida* spp., bacteria, and human placental cells were used (mean A_{650} range for non-*C. albicans* DNA, 0.000 to 0.018; Table 2).

Further, use of probes CT, CP, and CK resulted in positive EIA absorbance values only with PCR-amplified DNAs from *C. tropicalis*, *C. parapsilosis*, and *C. krusei*, respectively (mean A_{650}, 0.550 to 1.028). Results were considered positive when the A_{650} was greater than or equal to 0.060 (2 standard deviations above the mean A_{650} for the reagent blanks after subtraction of the reagent blank A_{650}). These results confirm the species specificity of these probes obtained when probes were radiolabeled and used in Southern blotting experiments with DNAs from 10 strains of *C. albicans*, 6 strains of *C. parapsilosis*, and 2 strains of *C. tropicalis* (17). Digoxigenin-labeled probe CK reacted relatively poorly with *C. krusei* DNA when hybridization was carried out for the standard 1-h incubation interval (data not shown) but gave strongly positive results when hybridization was conducted for 24 h (Table 2). The extended hybridization time used for probe CK resulted in higher, although negative, background absorbance values relative to values obtained when the standard 1-h incubation was used (Table 2). Therefore, a 24-h hybridization time was used for probe CK only. Probe CG reacted with *C.* (*Torulopsis*) *glabrata* DNA but also cross-reacted with *S. cerevisiae* DNA, even though hybridization was carried out for the standard 1-h incubation interval (Table 2). Therefore, all probes except CG demonstrated species specificity.

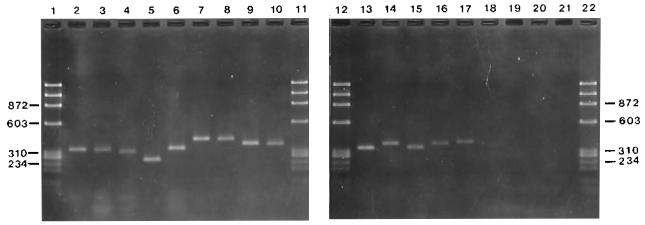

FIG. 1. EtBr-stained agarose gel of PCR products obtained from fungal DNA targets and controls by using primers ITS3 and ITS4. Lanes: 1, 11, 12, and 22, molecular size markers (*Hae*III-digested ϕX174 replicative-form DNA; fragment sizes are given in base pairs); 2 and 13, *C. albicans* H317 and B311, respectively; 3, *C. tropicalis*; 4, *C. parapsilosis*; 5, *C. guilliermondii*; 6, *C. krusei*; 7, *C.* (*Torulopsis*) *glabrata*; 8, *S. cerevisiae*; 9, *C. neoformans*; 10, *A. fumigatus*; 14, *A. flavus*; 15, *P. marneffei*; 16, *B. dermatitidis*; 17, *H. capsulatum*; 18, *S. aureus*; 19, *E. coli*; 20, *P. aeruginosa*; 21, human placental cell DNA.

TABLE 2. Species specificities of DNA probes analyzed by hybridization with PCR products in an EIA

Genomic DNA tested[a]	Mean A_{650}[b] for EIA detection of PCR products with probe:				
	CA	CT	CP	CK[c]	CG
C. albicans	**0.564**	0	0.008	0.026	0.002
C. tropicalis	0.018	**1.028**	0.008	0.021	0
C. parapsilosis	0.017	0.001	**0.550**	0.013	0.018
C. guilliermondii	0	0.001	0.001	0.013	0.002
C. krusei	0	0.001	0	**0.647**	0
C. (*Torulopsis*) *glabrata*	0	0.001	0	0.044	**1.847**
S. cerevisiae	0	0	0	0.054	**1.238**
C. neoformans	0	0	0	0.007	0.003
A. fumigatus	0	0	0	0	0
A. flavus	0	0	0	0	0
P. marneffei	0	0	0	0	0
B. dermatitidis	0	0	0	0	0.001
H. capsulatum	0	0	0	0	0
S. aureus	0	0	0	0	0.001
E. coli	0.002	0	0	0	0
P. aeruginosa	0	0	0	0	0.003
Human cell line	0	0	0	0	0.003

[a] One nanogram of purified DNA was PCR amplified as described in Materials and Methods. The data shown were compiled from five experiments.

[b] The mean of reagent blanks for a given probe was subtracted from each sample. The mean A_{650} of reagent blanks for all five probes ± the standard deviation was 0.046 ± 0.030 (n = 5). Values of ≥0.060 were considered positive (2 standard deviations above the mean A_{650} of reagent blanks after subtraction of the reagent blank A_{650}). Boldface type indicates positive hybridization results between DNA probe and PCR product.

[c] The hybridization time for all probes was 1 h, except that for probe CK, which was 24 h.

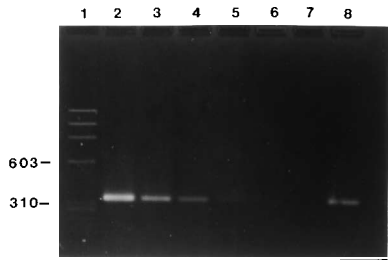

FIG. 2. EtBr-stained agarose gel detection of PCR-amplified DNA from *C. albicans* suspended in blood. Lanes: 1, molecular size markers (*Hae*III-digested ϕX174 replicative-form DNA; fragment sizes are given in base pairs); 2 to 6, PCR products derived from 2,000 to 0 *C. albicans* B311 blastoconidia suspended in blood (numbers of blastoconidia: lane 2, 2,000; lane 3, 200; lane 4, 20; lane 5, 2; lane 6, 0); 7, distilled-water template control; 8, PCR product from 1 ng of amplified *C. albicans* DNA.

Sensitivity of PCR product detection by EIA versus ethidium bromide-stained agarose gels. DNA from 10^1 to 10^4 *C. albicans* blastoconidia per ml of blood was isolated, purified, and PCR amplified as described in Materials and Methods. Amplified DNA was then detected following agarose gel electrophoresis and ethidium bromide (EtBr) staining (Fig. 2). The sensitivity of detection by EtBr staining was 20 cells per 0.2-ml sample or 10^2 cells per ml of blood. In comparison, Table 3 shows the results of microtitration plate EIA detection of amplified DNA derived from *C. albicans* blastoconidia suspended in blood by using digoxigenin-labeled *C. albicans*-specific probe CA. Samples that gave an A_{650} of 0.020 or greater were considered positive (2 standard deviations above the mean A_{650} for the reagent blank after subtraction of the reagent blank A_{650}). The limit of DNA detection by EIA was 2 *C. albicans* cells per 0.2 ml of test sample or 10 cells per ml of blood. Therefore, the sensitivity of detection of PCR-amplified *C. albicans* DNA by the microtitration plate EIA was 10-fold greater than that of detection by EtBr staining of agarose gels (compare Fig. 2 with Table 3).

EIA absorbance values for two or more *C. albicans* blastoconidia per 0.2 ml of blood were significantly higher than those for control samples containing no *C. albicans* blastoconidia ($P < 0.01$ or better). Negative controls (no primer or no DNA) gave significantly lower absorbance values (mean A_{650} of 0.009 or less) than samples containing blastoconidia (0.080 ± 0.012 to 0.823 ± 0.068, $P < 0.01$ or $P < 0.005$, $n = 3$). The absorbance values obtained by EIA for detection of *C. albicans* DNA with heterologous digoxigenin-labeled probes CT, CP, CK, and CG were negative (mean A_{650}, less than 0.010).

TABLE 3. EIA detection of PCR-amplified DNA from *C. albicans* blastoconidia suspended in blood

No. of *C. albicans* blastoconidia[a]	Mean A_{650}[b] for EIA with *C. albicans*-specific probe CA			
	Expt 1	Expt 2	Expt 3	Mean ± SD
2,000	0.901	0.783	0.785	0.823 ± 0.068[c]
200	0.488	0.523	0.689	0.567 ± 0.107[c]
20	0.193	0.356	0.131	0.227 ± 0.116[c]
2	0.066	0.088	0.087	0.080 ± 0.012[c]
0	0.009	0.009	0.008	0.009 ± 0.001
C. albicans DNA (1 ng)	0.464	0.440	0.348	0.417 ± 0.061[c]

[a] Known numbers of *C. albicans* B311 blastoconidia (10^1, 10^2, 10^3, and 10^4) were introduced into 1 ml of whole blood as described in Materials and Methods. A 0.2-ml aliquot was removed from each sample for DNA isolation, purification, and detection.

[b] The values shown were determined after subtraction of the mean A_{650} of reagent blanks for probe CA. The mean A_{650} for reagent blanks ± the standard deviation was 0.075 ± 0.010 ($n = 3$). Values of ≥0.020 were considered positive (2 standard deviations above the mean A_{650} of reagent blanks after subtraction of the reagent blank A_{650}).

[c] $P < 0.01$ or $P < 0.005$ by Student's t test versus the mean for samples containing no *C. albicans* blastoconidia or DNA.

DISCUSSION

We designed a PCR-based test to detect *Candida* sp. DNA in blood for the following clinically important reasons: (i) candidemia has been identified as an independent risk factor for predicting death, (ii) nosocomial bloodstream infections due to *Candida* spp. have risen from the eighth to the fourth most frequent cause of septicemia, and (iii) crude mortality in the presence of candidemia has been reported to be 57%, with 46% of these patients dying within 1 week of the onset of fungemia (27). Unlike urine or sputum, blood is a normally sterile fluid. Therefore, isolation of *Candida* sp. DNA from blood, particularly from that of persistently granulocytopenic patients, raises the suspicion of deep-seated infection (1). In addition, a PCR-based test for candidemia should be more sensitive than conventional blood culture methods since DNA from dead blastoconidia, as well as that from viable blastoconidia, is detected and the target sequence is amplified many-fold.

Previous reports described PCR amplification of *Candida* sp. DNA with primers selected from highly conserved single- or multiple-copy genes (4, 7, 15, 24). Gene targets included single-copy genes for heat shock protein 90 (7), actin (15), and lanosterol C_{14}-demethylase (3, 4) and a moderately repeated restriction fragment of *C. albicans* mitochondrial DNA, EO3 (24). To increase the sensitivity of detection of amplified DNA without use of nested PCR techniques (which add to the time required to perform PCR-based tests and to the risk of sample contamination due to additional sample manipulation), we chose universal fungal primers that are known not only to amplify high-copy-number (40 to 80 repeat copies per haploid genome) RNA genes (32, 36) but to amplify DNA from most, if not all, fungi (36). Although these primers are universal and highly conserved, some variations in amplification product size occur with their use (for example, fungal DNAs amplified with primers ITS3 and ITS4 gave amplicons ranging from 260 to 410 bp in the present study). However, precise identification of an individual fungus requires use of species-specific oligonucleotide probes or DNA sequencing. Combined use of universal fungal primers to give broad detection capabilities for all fungi, followed by use of genus- and species-specific probes, is an attractive approach to the diagnosis of fungal infections. We chose this approach to develop our microtitration plate EIA since identification of fungi with a battery of genus- and species-specific probes is particularly well suited to the clinical setting, multiple samples can be processed rapidly in duplicate or triplicate, and the process is amenable to automation, unlike Southern blotting.

Previous methods for the detection of PCR products used radiolabeled probes and Southern blotting or EtBr staining of agarose gels (4, 12, 24). Detection of PCR products in EtBr-stained gels has been shown by others to give 1-log-lower sensitivity than Southern blotting (24) and also was 1 log lower in sensitivity than our microtitration plate EIA (compare Fig. 2 and Table 3). Although ^{32}P-labeled *C. albicans*-specific probes have been used by others to increase test sensitivity (4, 7, 15, 24), radioisotopes are impractical for routine laboratory use because of their toxicity, expense, and short half-life. To

circumvent these disadvantages, we used nonisotopic, digoxigenin-labeled probes that specifically hybridized with amplified template DNA from *C. albicans*, *C. tropicalis*, *C. parapsilosis*, or *C. krusei*. One probe, CG, designed to detect *C.* (*Torulopsis*) *glabrata* DNA, hybridized with template DNA from *S. cerevisiae*. This result is not surprising, given the high degree of genetic relatedness of these two species on the basis of sequence analysis of rRNA genes (22). However, it does not mean that species-specific probes used to differentiate these two species cannot be designed from adjacent regions of the ITS region, and this should be attempted in the near future. It is clinically relevant, however, that our probes could differentiate *C.* (*Torulopsis*) *glabrata* and *S. cerevisiae* DNAs from those of the other *Candida* spp., since both *C. glabrata* and *S. cerevisiae* have been reported to be resistant to fluconazole (5).

Ours is the first report of a microtitration plate EIA used to detect *C. albicans* DNA following PCR amplification. Analysis of PCR products by EIA is easier and more rapid than that by Southern blotting, as conducted by others (13) and by our laboratory (unpublished observations). In addition, we hybridized PCR products with digoxigenin-labeled, species-specific probes and a biotinylated capture probe in the liquid phase before binding to microtitration plates since solution hybridization is considered to be 10 to 20 times faster than solid-phase hybridization (26, 31). Moreover, use of two separate probes (one biotinylated, one digoxigenin labeled) has been shown to contribute to more sensitive and specific detection of the PCR product (9).

Recently, Holloway et al. (9) reported that a T7 gene 6 exonuclease-digested PCR product was more easily detected in an EIA format than products that were heat denatured (i.e., 2-log-greater sensitivity after standard gel analysis and EtBr staining and more than 1-log-greater sensitivity after Southern blot analysis). In the present study, single-stranded DNA was prepared by heat denaturation of PCR amplicons since no improvement in sensitivity was observed with the T7 gene 6 method. In addition, heat denaturation was easier and required only 5 min to perform, in contrast to 30 min for exonuclease digestion. The exonuclease method may not have shown improved sensitivity in our EIA because of the amplicon size obtained in our experiments. The exonuclease technique was originally reported to be successful for amplicons smaller than 100 bp (9), whereas our amplicons were approximately 300 to 400 bp. This increased amplicon size may have interfered with the efficiency of the exonuclease method (8b). Also, the exonuclease method may conserve the probe since others have found that lower probe concentrations (<5 ng/ml) could be used after exonuclease digestion of target DNA with results comparable to those obtained after heat denaturation of DNA (8a).

To examine the reproducibility of our PCR assay, we prepared *C. albicans* template DNA from blood on three different days and found the sensitivity of detection to be two cells per 0.2 ml of blood in each experiment. The sensitivity of detection by the microtitration plate EIA was higher than that obtained by Miyakawa et al. (Three cells per 0.1 ml of blood [24]) or Holmes et al. (15 CFU/ml of blood [10]), who also used seeded blood but used Southern blotting to detect PCR products. Our microtitration plate assay is particularly attractive since in addition to high sensitivity relative to that reported by others, it has the advantage of not requiring radioactive probes or Southern blotting techniques for DNA detection.

Although detection of *Candida* spp. other than *C. albicans* in blood was not attempted in the present study, the specificity of the other *Candida* sp. probes tested in the EIA indicates their potential for detection and differentiation of other *Candida* spp. in blood.

We describe a rapid and simple method for detection of PCR products of *Candida* sp. DNA by a microtitration plate EIA that is easily adaptable to the clinical setting and to automation. Although progress needs to be made to simplify sample preparation, our methods for isolation and purification of *Candida* DNA from blood is no more complicated than those previously described (3, 10, 24). Further studies are under way to simplify DNA isolation and purification methods to make PCR-based detection of candidemia more rapid and practical in the clinical laboratory setting. Concurrently, studies are in progress to determine if the sensitivity and specificity obtained with seeded blood can be obtained with clinical specimens.

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