JOURNAL OF CLINICAL MICROBIOLOGY, Mar. 1997, p. 667–672 Vol. 35, No. 3
0095-1137/97/$04.00+0

Identification of Various Medically Important *Candida* Species in Clinical Specimens by PCR-Restriction Enzyme Analysis

GIULIA MORACE,* MAURIZIO SANGUINETTI, BRUNELLA POSTERARO, GIULIANA LO CASCIO, AND GIOVANNI FADDA

Istituto di Microbiologia, Università Cattolica Sacro Cuore, 1-00168 Rome, Italy

Received 10 June 1996/Returned for modification 17 July 1996/Accepted 2 December 1996

A single primer pair amplifying a cytochrome P-450 lanosterol-14α-demethylase (L1A1) gene fragment that encodes a highly conserved region was used to detect yeast DNA in clinical specimens. Positive PCR products were obtained from genomic DNAs of *Candida albicans*, *C. parapsilosis*, *C. tropicalis*, *C. guilliermondii*, *C. krusei*, *C.* (*Torulopsis*) *glabrata*, and *C. kefyr*. No human, bacterial, or parasitic DNA was amplified. The sensitivity was evaluated for *C. albicans* genomic DNA by using various DNA concentrations (200 pg to 2 fg). The amplified DNAs of *Candida* species with unknown P-450 L1A1 gene sequences were subcloned and sequenced. Identification at the species level was achieved by digestion of the PCR products with different restriction enzymes. A specific restriction enzyme analysis pattern was determined for each species investigated. Subsequently, we used PCR to detect specific yeast DNA directly with clinical specimens such as blood and bronchoalveolar lavage specimens. After appropriate treatment, the specimens were processed by PCR and the results were compared with those obtained by traditional diagnostic procedures such as cultures and serology. Although preliminary, the PCR results seem to correlate well, at least for blood, with those of antigen detection assays and traditional blood cultures, with a better and earlier detection of candidemia.

The hematogenous spread of yeasts occurs frequently in immunocompromised patients and leads to severe life-threatening disseminated mycoses. Conventional blood cultures are not very effective in detecting yeast growth and take too long to become positive (26), although great progress has been made in recent years (7). Alternative procedures such as the detection of circulating antigens, enzymes, or metabolic products (17, 26, 31, 33) lack sensitivity and, to some extent, specificity, so diagnosis can be delayed or in most cases is obtained only at autopsy (4, 8, 10, 25, 26). Since candidosis is the most common disseminated fungal infection in leukemia or cancer patients, an early diagnosis of candidemia is therefore of great importance for patient care, especially if it leads to the selection of a rapid, species-specific antifungal therapy, which could likely reduce mortality (8).

Amplification of a small amount of yeast-specific DNA in clinical specimens by PCR technology provides a more rapid and sensitive approach to the diagnosis of disseminated candidosis. Since 1990, several studies have been published on the detection of fungal DNA in clinical specimens (blood and bronchoalveolar lavage specimens) by the use of PCR. Detection of *Candida* DNA has been obtained by PCR amplification of ribosomal (9, 25, 29, 32), actin (14), heat shock protein 90 (6), and chitin synthase (12) genes. For filamentous fungi, PCR amplification of fungal DNA in clinical specimens has been successfully used for *Aspergillus*, *Penicillium*, and *Verticillium* spp. (18–20, 22, 30). Most of these studies, especially those on yeast DNA amplification, were limited by the presence of unspecific amplified products (2), by the inability to amplify DNA from *Candida* species other than *Candida albicans*, or by the small number of *Candida* species whose DNA could be amplified (3). Specific amplification of DNA from numerous important yeasts, including *Cryptococcus neoformans* and *Trichosporon beigelii*, resulted from the use of primer sets which amplify a fragment of the cytochrome P-450 lanosterol-14α demethylase (L1A1) gene (2, 3). This gene is fungus specific, and its product catalyzes the conversion of lanosterol to ergosterol, which is an essential component of the fungal plasmalemma and the main target of most antifungal drugs.

In the present study, a pair of primers, selected from the constant region of the P-450 L1A1 gene, which codifies for the active site of the enzyme, amplified a 300- to 350-bp segment of this gene from the genomic DNAs of numerous isolates of the *Candida* species *C. albicans*, *C. tropicalis*, *C. krusei*, *C.* (*Torulopsis*) *glabrata*, *C. guilliermondii*, *C. parapsilosis*, and *C. kefyr*. Moreover, restriction enzyme analysis (REA) of the PCR products allowed us to identify amplified *Candida* DNA at the species level. The diagnostic usefulness of these primers was studied by detecting and identifying *Candida* DNA in blood and bronchoalveolar lavage specimens from immunocompromised and surgical patients.

MATERIALS AND METHODS

Isolates. Yeasts were isolated from clinical specimens submitted to our laboratory for suspected fungal infections. The most representative species, in order of frequency of isolation, were *C. albicans*, *C. glabrata*, *C. krusei*, *C. tropicalis*, *C. parapsilosis*, *C. kefyr*, and *C. guilliermondii*. They were identified by the germination tube test in bovine fetal serum, with the yeast biochemical cards of the Vitek Automicrobic System (BioMerieux, Rome, Italy), and by micromorphology on rice extract agar. The total numbers of yeast isolates used in the study were 39 *C. albicans*, 20 *C. glabrata*, 20 *C. krusei*, 15 *C. tropicalis*, 10 *C. parapsilosis*, 10 *C. kefyr*, and 5 *C. guilliermondii* isolates. In addition, *C. albicans* CDC B 385 (Centers for Disease Control and Prevention [CDC], Atlanta, Ga.), *C. tropicalis* CBS 94 (Centraalbureau voor Schimmelcultures [CBS], Baarn, The Netherlands), *C. glabrata* CBS 138, and *C. krusei* CBS 573 were tested as type strains.

DNA extraction. Yeast cells were cultured on YEPD broth (1% yeast extract, 2% peptone, 2% dextrose) and were incubated overnight at 37°C under shaking conditions (240 rpm). The yeast cells were collected by centrifugation (1.5 ml of the broth culture at 10,000 × *g* for 5 min), suspended in 1 ml of 1 M sorbitol, centrifuged again, and resuspended in 1 ml of 1 M sorbitol–50 mM phosphate buffer (pH 7.5) containing 2% β-mercaptoethanol and 2 mg of yeast lytic enzyme (ICN, Aurora, Ohio). After 1 h of incubation at 37°C and microscopic visualization of the spheroplasts, the cells were centrifuged at 8,000 × *g* for 5 min, suspended in 0.5 ml of 50 mM EDTA (pH 8.0)–0.2% sodium dodecyl sulfate, and incubated at 70°C for 30 min. After the addition of 5 M potassium acetate (50 μl;

* Corresponding author. Mailing address: Istituto di Microbiologia, Facoltà di Medicina e Chirurgia "A. Gemelli," Università Cattolica del Sacro Cuore, Largo Francesco Vito 1-00168 Rome, Italy. Phone: 39-6-30154964. Fax: 39-6-3051152.

pH 5.4), the suspension was left at 0°C for 30 min and then centrifuged at 10,000 × *g* for 20 min. The supernatant was treated with RNase (10 μg/ml for 30 min at 37°C), and then the DNA was extracted with an equal volume of chloroform-isoamyl alcohol (24/1; three times). This DNA, after purification by passage through a Sephadex G50 column, was precipitated with cold ethanol at −70°C for 30 min and centrifuged at 10,000 × *g* for 20 min. The pellet was then suspended in 100 μl of TE (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]). Human, bacterial, and parasitic DNAs were extracted from cutaneous biopsy specimens, *Escherichia coli* and *Pseudomonas aeruginosa*, and *Trichomonas vaginalis*, respectively by standard procedures (21, 27).

PCR assay. The synthetic oligonucleotides used were primer $P450_1$ (base pairs 1021 to 1043; 5′-ATGACTGATCAAGAAATYGCTAA-3′), primer $P450_2$ (base pairs 1370 to 1351; 5′-TAACCTGGAGAAACYAAAAC-3′), and the internal probe P450 int (base pairs 1063 to 1082; 5′-ATGGGTGGTCAACATACTTC-3′). The base pair numbers refer to the locations within the published P-450 L1A1 gene sequence of *C. albicans* (16).

DNA (200 pg) was added to 100 μl of the PCR master mixture: 10 μl of a 10× PCR buffer (100 mM Tris-HCl [pH 8.3], 500 mM KCl, 15 mM $MgCl_2$), 16 μl of a mixture of 200 μM each deoxynucleoside triphosphate (equimolar concentrations of dATP, dCTP, dGTP, and dTTP), 50 ng of each primer, and 2.5 U of *Taq* DNA polymerase (Boehringer Mannheim, Mannheim, Germany), with the remaining volume made up with sterile distilled water. The PCR was performed in a thermal cycler (Gene Amp PCR System 9600; Perkin-Elmer). The first cycle included 3 min of denaturation at 94°C, 1 min of annealing at 49°C, and 1 min and 30 s of primer extension at 72°C, followed by 40 cycles of 45 s of denaturation at 94°C, 1 min of annealing at 49°C, and 1 min and 30 s of extension at 72°C.

The PCR products were electrophoresed in an agarose gel (2%) for 1 h and 30 min at 70 V at room temperature in TAE buffer (40 mM Tris acetate, 1 mM EDTA [pH 8.4]), stained with ethidium bromide, and visualized with UV light (302 nm). To assess sensitivity, serial dilutions of *C. albicans* DNA (200 pg to 2 fg) were used in the PCR assay. The amplified electrophoresed fragments were transferred onto a nylon membrane by standard procedures (21) and were hybridized with the P450 int 3′ digoxigenin-labeled probe according to the manufacturer's instructions (DIG oligonucleotide 3′ end labeling kit; DIG DNA detection kit from Boehringer Mannheim).

Cloning and sequencing of the PCR products. The PCR products of *C. parapsilosis*, *C. kefyr*, and *C. guilliermondii* were cloned in plasmid pUC19 at the *Sma*I site and were sequenced in a Sequigen apparatus (Bio-Rad) by using the Sequenase, version 2.0, sequencing kit (U.S. Biochemicals) with [α-^{35}S]dATP (Amersham) according to the manufacturer's instructions.

REA procedure. The PCR products were subjected to REA by using *Sau*3A, *Hinc*II, and *Nsi*I according to the manufacturer's instructions. After digestion, the DNAs were subjected to electrophoresis in an agarose gel (4% gel [3% SeaKem GTG and 1% Nusieve] in TAE buffer) at 70 V for 2 h at room temperature and were then stained with ethidium bromide (0.5 μg/ml) and photographed with UV light.

Preparation of clinical specimens. Bronchoalveolar lavage specimens (1 ml; 20 specimens from 20 leukemia or AIDS patients) were centrifuged at 10,000 × *g* for 5 min. The supernatant was discarded and the pellet (washed two times with phosphate-buffered saline [PBS]) was resuspended in 1 ml of PBS and stored at 4°C until it was used for DNA extraction. Blood samples (3 ml; 21 samples from 18 patients) were collected in tubes containing EDTA and were then diluted 1:1 with sterile PBS (pH 7.2) and added to 3 ml of Ficoll (Diatrizoate-Ficoll; EUROBIO, Les Ulis, France). The samples were centrifuged at 1,200 × *g* for 30 min to collect leukocytes and yeast cells. After two washes in PBS (30 ml), the cells (leukocytes and yeast cells) were resuspended in 1 ml of PBS and were stored at 4°C until they were used. Extraction of yeast DNA from the processed clinical specimens was done by the method of Rand et al. (25). Briefly, samples were centrifuged at 10,000 × *g* for 10 min in an Eppendorf microcentrifuge, and the pellets were resuspended in 0.5 ml of lysing solution (0.5% Nonidet P-40, 0.5% Tween 20, and 0.5% Triton X-100 in double-distilled H_2O) and warmed at 37°C for 15 min. After centrifugation (10,000 × *g* for 30 min), the pellets were resuspended in 0.5 ml of 1 M sorbitol–50 mM phosphate buffer (pH 7.5) containing 2% β-mercaptoethanol and 2 mg of yeast lytic enzyme (ICN), and the mixture was incubated for 2 h at 37°C. The spheroplasts were then lysed with proteinase K and sodium dodecyl sulfate (0.5 mg/ml and 0.2%, respectively). After two extractions with phenol-chloroform-isoamyl alcohol (25:24:1), the DNA was purified by passage through a Sephadex G50 column and was precipitated with cold ethanol. The pellet was suspended in 20 μl of sterile distilled water, and 10 μl was used to perform the PCR as described above. Blood samples from six healthy people, including three of the authors, taken two times at a 15-day interval were used as negative controls for processing of blood for PCR.

Conventional diagnostic methods. All 20 bronchoalveolar lavage specimens were also processed and cultured for fungi by standard procedures (23). Fifteen blood samples from 13 patients were cultured in aerobic and anaerobic bottles (Plus/aerobic/S* medium and Plus/anaerobic/F* medium, respectively) for the BACTEC 9240 automated system (Becton Dickinson, Cockeysville, Md.), and all positive blood cultures were examined microscopically and plated on Bacto Candida Growth medium (Difco) and Sabouraud dextrose agar. Assays for the detection of antigen in 19 serum samples were performed with the Candida Tec kit (Ramco Laboratories, Houston, Tex.). Cultures and serological assays were also performed, and the results were compared with those of PCR of blood.

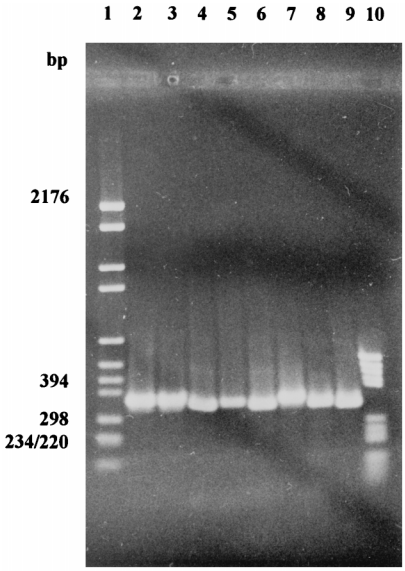

FIG. 1. PCR amplicons from genomic DNAs of *C. albicans* (lane 2), *C. tropicalis* (lane 3), *C. guilliermondii* (lane 4), *C. parapsilosis* (lane 5), *C. kefyr* (lane 6), *C. krusei* (lane 7), *C. glabrata* (lane 8), and *C. neoformans* (lane 9). Lanes 1 and 10, molecular weight markers (Boehringer Mannheim marker VI and marker V, respectively).

Nucleotide sequence accession numbers. The nucleotide sequence data reported in this paper will appear in the EMBL, GenBank, and DDBJ nucleotide sequence databases under the accession numbers X97680 CGP450L (*C. guilliermondii*), X97682 CKP450L (*C. kefyr*), and X97681 CPP450L (*C. parapsilosis*).

RESULTS

Under the experimental conditions described above, we obtained positive PCR products with DNAs extracted from *C. albicans*, *C. tropicalis*, *C. glabrata*, *C. krusei*, *C. parapsilosis*, *C. kefyr*, *C. guilliermondii*, and *C. neoformans* (Fig. 1), as well as *Saccharomyces cerevisiae*, *Candida lusitaniae*, *Candida rugosa*, *Candida lambica*, and *Blastoschizomyces capitatus*. No human, bacterial, or parasitic DNAs were amplified. The primers used were unable to amplify DNA extracted from *Yarrowia lipolytica*, *Rhodotorula rubra*, *Rhodotorula glutinis*, *Rhodotorula minuta*, *Aspergillus fumigatus*, *Aspergillus flavus*, *Alternaria alternata*, and *Cladosporium* sp.

The sensitivity of the PCR assay was evaluated with DNA at various concentrations (from 200 pg to 2 fg) of *C. albicans* genomic DNA. Our primer set was able to amplify 200 fg of *C. albicans* DNA, and Southern blot analysis with the P450 int probe confirmed the specificity of the amplicon (Fig. 2).

The sizes of the PCR amplicons obtained from yeast species DNAs corresponded to the sizes that were expected not only for the species for which the sequences of the amplified fragments are known but also for *C. guilliermondii*, *C. kefyr*, and *C. parapsilosis*. The amplified products from these three species were cloned and sequenced; the sequences of the fragments (Fig. 3) were compared with the published gene sequences of *C. albicans* (16), *C. tropicalis* (5), *C. krusei* (3), *C. glabrata* (3), and *S. cerevisiae* (13); this comparison resulted in good percentages of homology between all species. The closest homologies observed were 91.3% between *C. albicans* and *C. guilliermondii*, 84.8% between *C. kefyr* and *S. cerevisiae*, and 69.4% between *C. parapsilosis* and *C. tropicalis*. Knowledge of the sequences of the PCR amplicons enabled us to perform REA with appropriate restriction enzymes (Fig. 4 and 5); this analysis resulted in the identification of all seven *Candida* species

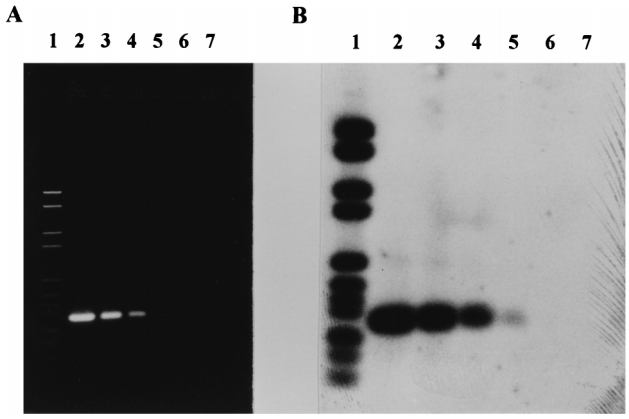

FIG. 2. Sensitivity of the PCR assay by agarose gel electrophoresis (A) or Southern blotting (B). Lanes 1, digoxigenin-labeled Boehringer Mannheim marker VI; lanes 2 through 7, PCR amplicons obtained from serial dilutions of *C. albicans* genomic DNA (from 200 pg to 2 fg).

investigated by the species-specific REA pattern (Table 1). Concerning the other three species of *Candida* whose DNAs were amplified by our primer set, the products that we obtained were smaller than those mentioned above; moreover, we tested only one isolate of each species, so further investigations need to be done with large numbers of isolates of these species.

When PCR was applied to the clinical specimens, we obtained positive results for 15 blood samples and 6 bronchoalveolar lavage specimens. Most of the patients were at risk for fungal infections because of their underlying diseases or conditions (leukemia, organ transplant, or AIDS), and a few were surgical patients. REA of the amplified fragments enabled us to identify the DNA at the species level. In Table 2, the results obtained by PCR-REA of blood are compared with culture results for blood samples submitted by clinicians and antigen detection assay results, if they were available. PCR gave more positive results than blood culture, and a better correlation was obtained by comparison of positive results by PCR and positive results by antigen detection assays. With the exception of two patients in whose blood *C. kefyr* was detected by PCR-REA and the patient in whose blood *C. guilliermondii* was detected by PCR-REA, all patients whose blood was positive by PCR and with negative blood cultures or for whom blood cultures were not done had antigen titers of ≥1:4; these titers can be considered an expression of possible disseminated candida infection. Negative blood culture results could be due either to the use of a nonoptimal system or possibly to the fact that insufficient numbers of yeast cells were inoculated into the bottles. All blood samples obtained from healthy people gave negative PCR results. For bronchoalveolar lavage specimens the PCR results were compared with culture results, and the results for all except two of the samples correlated well. For the first of these samples, the PCR result was positive for *C. albicans* DNA but the culture gave no growth, but a second bronchoalveolar lavage specimen from the same patient not processed by PCR grew *C. albicans* 7 days later. For the second sample, PCR results were negative, but *C. albicans* was isolated on culture. For the other four samples, the PCR products identified the organisms in the samples as *C. glabrata*, *C. kefyr*, *C. krusei*, and *C. tropicalis* by REA. The same species were isolated and identified by standard procedures from the cultures.

A

1	ATG	ACT	GAT	CAA	GAA	ATT	GCT	AAT	CTT	TTA	ATC	GTA	ATT	CTT	ATG	GGT	GGT	CAA	CAT	ACT	TCT	ACT	TCT
	M	T	D	Q	E	I	A	N	L	L	I	V	I	L	M	G	G	Q	H	T	S	T	S
70	GCT	TGG	TTC	TTG	TTA	CAT	TTA	GGT	GAA	AAA	CCT	CAT	TTA	CAA	GAT	GTT	ATT	TAT	CAA	GAA	GTT	GTT	GAA
	A	W	F	L	L	H	L	G	E	K	P	H	L	Q	D	V	I	Y	Q	E	V	V	E
139	TTA	TTG	AAA	GAA	AAA	GGT	GGT	GAT	TTG	AAT	GAT	TTG	ACT	TAT	GAA	GAT	TTA	CAA	AAA	TTA	CCA	TCA	GTC
	L	L	K	E	K	G	G	D	L	N	D	L	T	Y	E	D	L	Q	K	L	P	S	V
208	AAT	AAC	ACT	ATT	AAG	GAA	ACT	CTC	AGA	ATG	CAT	ATG	CCA	TTA	CAT	TCT	ACT	TTT	AGA	AAA	GTT	ACT	AAC
	N	N	T	I	K	E	T	L	R	M	H	M	P	L	H	S	T	F	R	K	V	T	N
277	CCA	TTA	AGA	TCA	CCG	AAC	AAA	TAT	ATG	TCA	AGT	CAT	ACG	GTG	TAT	ATG	TTT	AAG	TTT	CTC			
	P	L	R	S	P	N	K	Y	M	S	S	H	T	V	Y	M	F	K	F	L			

B

1	ATG	ACT	GAT	CAA	GAA	ATT	GCT	AAT	CTT	TTA	AGC	ATT	CTT	ATG	GGT	GGT	CAA	CAT	ACT	TCT	GCT	GCT	ACC
	M	T	D	Q	E	I	A	N	L	L	S	I	L	M	G	G	Q	H	T	S	A	A	T
70	TCC	GCT	TGG	GCT	ATT	TTG	CAC	TTG	GCT	GAA	AGA	CCA	GAT	GTT	CAA	CAA	GAA	TTA	TAC	GAA	GAA	CAA	ATG
	S	A	W	A	I	L	H	L	A	E	R	P	D	V	Q	Q	E	L	Y	E	E	Q	M
139	AGA	GTC	TTG	GAT	AAT	GGT	AAG	AAA	GAA	CTA	ACC	TAT	GAC	TTA	TTG	CAA	GAA	ATG	CCT	TTC	TTG	AAC	CAA
	R	V	L	D	N	G	K	K	E	L	T	Y	D	L	L	Q	E	M	P	F	L	N	Q
208	ACT	ATC	AAG	GAA	ACC	CAT	AGA	TTA	CAT	CAC	CCA	TTG	CAT	TCT	TTG	TTC	CGT	AAA	GTT	ATG	AAC	GAT	ATG
	T	I	K	E	T	L	R	L	H	H	P	L	H	S	L	F	R	K	V	M	N	D	M
277	CCT	GTT	CCA	AAC	ACA	TCT	TAT	GTT	GTT	CCA	AAA	GGT	GAT	CAT	GTT	TTA	GTT	TCT	CCA	GGT	TA		
	P	V	P	N	T	S	Y	V	V	P	K	G	D	H	V	L	V	S	P	G			

C

1	ATG	ACT	GAT	CAA	GAA	ATT	GCT	AAT	GTT	TTA	ATT	GGT	ATT	TTG	ATG	GGA	GGA	CAG	CAT	ACT	AGT	GCC	ACT
	M	T	D	Q	E	I	A	N	V	L	I	G	I	L	M	G	G	Q	H	T	S	A	T
70	ACA	TCT	GCT	TGG	TTT	TTG	TTG	CAT	TTG	GCT	GAG	AAG	CAC	CAT	TTA	CAA	GAT	GAA	TTG	TAT	CAA	GAG	GTT
	T	S	A	W	F	L	L	H	L	A	E	K	H	H	L	Q	D	E	L	Y	Q	E	V
139	CTT	AAT	GCC	TTA	TCT	GGT	AAA	GGT	GGC	AAC	TTG	GAT	GAT	TTA	TCG	TAT	GAG	GAT	TTG	CAA	CAA	ATG	CCA
	L	N	A	L	S	G	K	G	G	N	L	D	D	L	S	Y	E	D	L	Q	Q	M	P
208	TTG	GTT	AAC	TTC	AGA	AAA	GTT	GTT	TCT	CCC	TTG	GTT	GTG	CCA	AAC	TTA	CAT	TCC	ATA	TTC	AGA	AAA	GTT
	L	V	N	F	R	K	V	V	S	P	L	V	V	P	N	L	H	S	I	F	R	K	V
277	GTT	TCT	CCC	TTG	GTT	GTG	CCA	AAC	ACG	AAT	AAT	ATT	TTC	GAA	AAA	GCT	CAT	AAT	GTT	TTA	GTT	TCT	CCA
	V	S	P	L	V	V	P	N	T	N	N	I	F	E	K	A	H	N	V	L	V	S	P
346	GGT	TA																					
	G																						

FIG. 3. Nucleotide and deduced amino acid sequences of the PCR-amplified fragments of the P-450 L1A1 genes from *C. guilliermondii* UCSC CG001 (A), *C. kefyr* UCSC Cke005 (B), and *C. parapsilosis* UCSC CP010 (C).

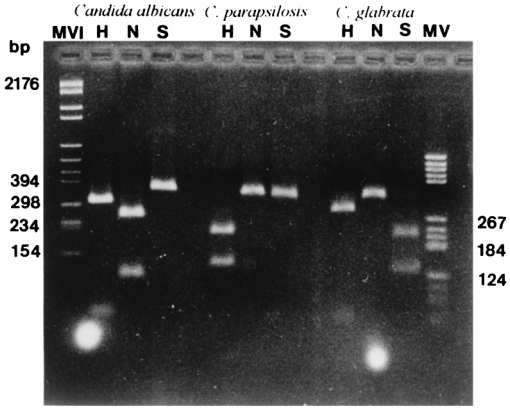

FIG. 4. Patterns of amplified products of three *Candida* spp. digested with *Hin*cII (lanes H), *Nsi*I (lanes N), and *Sau*3A (lanes S). Lane MVI, Boehringer Mannheim marker VI; lane MV, Boehringer Mannheim marker V.

DISCUSSION

The increasing incidence of fungemia due to a broad variety of yeast species in immunocompromised patients and the poor sensitivity of conventional blood cultures for detecting yeast growth have made it necessary to use alternative approaches for the early detection and identification of the causative yeast species (8, 10, 26, 31, 33). Several PCR approaches have been used for this purpose (2, 3, 6, 9, 12, 14, 19, 29, 32), and according to their sensitivities and specificities, each of them seems promising.

In our study, we used a single primer pair specifically designed to amplify a DNA fragment of 300 to 350 bp of the highly conserved region of the P-450 L1A1 gene. Its enzymatic product catalyzes the conversion of lanosterol to ergosterol, an important component of the fungal membrane. Moreover, most of the antifungal drugs, especially the azole derivatives, act on this biosynthetic pathway.

Our amplicons include the nucleotide sequence which encodes the amino acid region defined as distal helix I (residues 292 to 327) of the enzyme (1). This helix, which constitutes part of the active site, is a highly conserved region, and an amino acid substitution in this region could result in a loss of enzymatic activity, as has been demonstrated by Ishida et al. (11) for *S. cerevisiae*. In fact, a change in amino acid residue 310 causes a rotational shift of distal helix I, with the subsequent interaction of the His-317 residue with the heme iron (11). A point mutation in this region could be implied in the resistance to azole derivatives of medically important yeasts, and we are evaluating the possibility that our PCR assay could be useful for detecting such point mutations by studying a large number of *C. albicans* isolates with different patterns of susceptibility to the azoles.

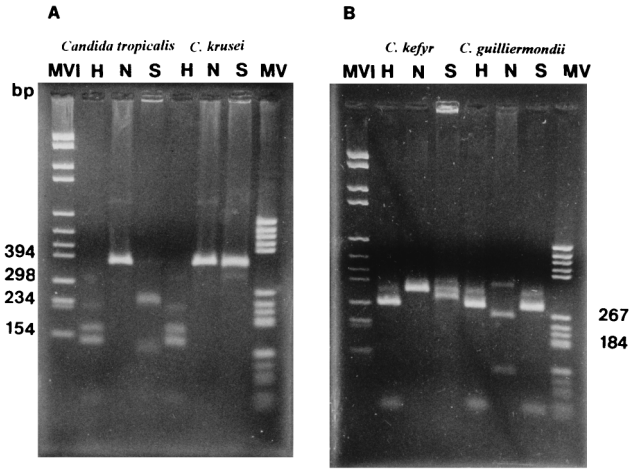

FIG. 5. Patterns of amplified products of four *Candida* spp. digested with *Hin*cII (lanes H), *Nsi*I (lanes N), and *Sau*3A (lanes S). Lanes MVI, Boehringer Mannheim marker VI; lanes MV, Boehringer Mannheim marker V.

TABLE 1. REA patterns obtained from the seven *Candida* species amplicons according to the different lengths of the fragments

Species	Fragment size (bp)			
	Uncut amplicon	*Hin*cII	*Nsi*I	*Sau*3A
C. albicans	350	300, 50	243, 107	343
C. glabrata	343	290, 53	343	213, 130
C. guilliermondii	336	283, 53	240, 96	277, 52
C. kefyr	338	288, 50	338	306
C. krusei	350	155, 139, 56	350	343
C. parapsilosis	350	214, 136	350	343
C. tropicalis	350	161, 138, 51	350	231, 114

The primers were able to amplify DNA from a great variety of yeast species, but not those from *Rhodotorula* spp., *Y. lipolytica*, and filamentous fungi, probably because they are distantly related to the genus *Candida*. The PCR product alone did not, however, give information about the species, and identification of the causative yeast species is important for therapeutic approaches, in view of the possible existence of naturally resistant species (24).

We therefore studied the possibility of identifying our amplicons at the species level. For *C. albicans*, *C. tropicalis*, *C. glabrata*, and *C. krusei*, knowledge of the nucleotide gene sequence that is amplified (3, 5, 16) allowed us to use restriction enzymes that are useful for obtaining species-specific REA patterns. For the other clinically relevant *Candida* species amplified, *C. kefyr*, *C. parapsilosis*, and *C. guilliermondii* (15, 28), the corresponding P-450 L1A1 gene fragments were cloned and sequenced to define a panel of restriction enzymes which allowed for the identification of the organisms to the species level. The close homologies encountered among the seven major *Candida* species studied allowed us to use the same panel of enzymes.

Our PCR approach demonstrated good sensitivity (200 fg of yeast DNA detected) and, with an additional step (REA procedure), allowed us to identify all seven species. It could therefore be considered useful for detecting and identifying candidal DNA direct from clinical specimens.

We therefore tested it with blood and obtained interesting results. Although the results are only preliminary, they indicate that PCR is more sensitive than blood culture for detecting yeast, especially in febrile patients unresponsive to antibiotic treatment, as were most of the patients whose blood was investigated. In this case, clinicians, especially hematologists, begin empiric antifungal treatment with amphotericin B. We were able to detect *C. albicans* DNA from a patient whose blood was culture positive for *P. aeruginosa*, which could have masked the detection of the yeast by culture. In addition, a few patients treated with antimycotic drugs on the basis of the positive PCR results showed marked clinical improvement, and for one patient (patient 14) a complete cure was achieved. These results are very preliminary and should be confirmed

TABLE 2. Comparison of results for blood processed by PCR, traditional blood cultures, and antigen detection assays

Patient no.	PCR result	REA result	Blood culture result	Antigen titer	Yeast(s) in other specimens
1	+	*C. glabrata*	Negative	1:4	None
2	+	*C. parapsilosis*	*C. parapsilosis*	Negative	None
3					
First specimen	+	*C. albicans*	Negative	1:8	*C. albicans*
Second specimen[a]	+	*C. albicans*	Negative	ND[b]	
4	+	*C. albicans*	*P. aeruginosa*	1:4	None
5	+	*C. tropicalis*	ND	1:4	None
6	+	*C. kefyr*	Negative	1:4	None
7	+	*C. kefyr*	Negative	1:2	None
8	+	*C. albicans*	Negative	1:8	ND
9	+	*C. albicans*	ND	1:8	*C. krusei* and *C. tropicalis*
10	−		Negative	Negative	*C. albicans*
11	−		Negative	Negative	*C. albicans*
12					
First specimen	−		ND	Negative	*C. rugosa*
Second specimen	+	*C. guilliermondii*	ND	Negative	
13	+	*C. krusei*	*C. krusei*	Negative	*C. krusei*
14					
First specimen	+	*C. kefyr*	Negative	1:2	ND
Second specimen	+	*C. kefyr*	Negative	1:2	
15	−		ND	1:2	*C. albicans*
16	−		ND	Negative	*C. albicans*
17	+	*C. glabrata*	*C. glabrata*	Negative	*C. glabrata*
18	+	*C. albicans*	*C. albicans*	ND	*C. albicans*

[a] The second blood specimens were obtained 7 days later.
[b] ND, not done.

with a larger number of patients. The occurrence of the yeast species detected in blood by PCR reflects our epidemiological data for blood cultures positive for yeasts; except for *C. kefyr*, this finding is unusual and could be considered a transient candidemia due to the inoculation of the yeast into the bloodstream from the skin (the patients were receiving parenteral nutrition) or during the operation (all the three *C. kefyr*-positive subjects were surgical patients). Colonization with yeasts (Table 2) does not seem to have an effect on the PCR results, as demonstrated for PCR-negative patients 10, 11, 15, and 16 with mucocutaneous candidosis or some PCR-positive patients (patients 2, 4, 5, 6, and 7) who were not colonized. For bronchoalveolar lavage specimens, the PCR results were comparable to those obtained by traditional cultures, being discordant only for two patients: one patient was PCR positive and culture negative, and one patient was PCR negative and culture positive.

The processing of blood specimens by the addition of Ficoll improves the detection of fungal DNA, since it eliminates erythrocytes, which could have an inhibitory effect on PCR assays, and separates yeast cells, as demonstrated with seeded specimens, in the same fraction of leukocytes.

However, the occurrence of false-positive or false-negative PCR results must be investigated with a larger number of specimens from patients at risk of developing disseminated fungal infections. To validate our PCR-REA system, we are performing a study with a larger number of patients with hematological malignancies who have fever of unknown origin and who are still febrile after 3 days of treatment with broad-spectrum antibiotics. These patients will be enrolled in the study before they receive empiric antifungal therapy and will be followed during the course of antifungal treatment by PCR of blood samples, and cultures of blood with Plus/fungal medium bottles from Becton Dickinson will be performed simultaneously. There is also the need to differentiate transient from sustained candidemias and to apply our PCR assay to the detection of *C. neoformans* in cerebrospinal fluid and blood specimens from patients under antimycotic treatment with persistent titers of antigen in cerebrospinal fluid and blood and negative cultures.

ACKNOWLEDGMENTS

This work was supported by contract no. 95.00402.PF39, targeted Project ACRO of the National Research Council (CNR).

REFERENCES

1. **Boscott, P. E., and G. H. Grant.** 1994. Modeling cytochrome P450 14α demethylase (*Candida albicans*) from P450cam. J. Mol. Graphics **12:**185–192.
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 *Candida 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. Bille, 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. **Butler, K. M., and C. J. Baker.** 1988. *Candida*: an increasingly important pathogen in the nursery. Pediatr. Clin. N. Am. **35:**543–563.
5. **Chen, C., V. F. Kalb, T. G. Turi, and J. C. Loper.** 1988. Primary structure of the cytochrome P-450 lanosterol 14α-demethylase from *Candida tropicalis*. DNA **7:**617–626.
6. **Crampin, A. C., and R. C. Matthews.** 1993. Application of the polymerase chain reaction to the diagnosis of candidosis by amplification of an HSP 90 gene fragment. J. Med. Microbiol. **39:**233–238.
7. **Geha, D. J., and G. D. Roberts.** 1994. Laboratory detection of fungemia. Clin. Lab. Med. **14:**83–97.
8. **Fraser, V. J., M. Jones, J. Dunkel, S. Storfor, 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.
9. **Holmes, A. R., R. D. Cannon, M. G. Shepherd, and H. F. Jenkinson.** 1994. Detection of *Candida albicans* and other yeasts in blood by PCR. J. Clin. Microbiol. **32:**228–231.
10. **Horn, R., B. Wong, T. E. Kiehn, and D. Armstrong.** 1985. Fungemia in a cancer hospital: changing frequency, earlier onset, and results of therapy. Rev. Infect. Dis. **7:**646–655.

11. **Ishida, N., Y. Aoyama, R. Hatanaka, Y. Oyama, S. Imajo, M. Ishiguro, T. Oshima, H. Nakazato, T. Noguchi, U. S. Maitra, V. P. Mohan, D. B. Sprinson, and Y. Yoshida.** 1988. A single aminoacid substitution converts cytochrome $P450_{14DM}$ to an inactive form cytochrome $P450_{SG1}$: complete primary structures deduced from cloned DNAs. Biochem. Biophys. Res. Commun. **155:**317–323.
12. **Jordan, J. A.** 1994. PCR identification of four medically important *Candida* species by using a single primer pair. J. Clin. Microbiol. **32:**2962–2967.
13. **Kalb, V. F., C. W. Woods, T. G. Turi, C. R. Dey, T. R. Sutter, and J. C. Loper.** 1987. Primary structure of the P450 lanosterol demethylase gene from *Saccharomyces cerevisiae*. DNA **6:**529–537.
14. **Kan, V. L.** 1993. Polymerase chain reaction for the diagnosis of candidemia. J. Infect. Dis. **168:**779–783.
15. **Kiehn, T. E., F. F. Edwards, and D. Armstrong.** 1980. The prevalence of yeasts in clinical specimens from cancer patients. Am. J. Clin. Pathol. **73:** 518–521.
16. **Lai, M. H., and D. R. Kirsch.** 1989. Nucleotide sequence of cytochrome P450 L1A1 (lanosterol 14α-demethylase) from *Candida albicans*. Nucleic Acids Res. **17:**804.
17. **Lemieux, C., G. St. Germain, J. Vincelette, L. Kaufman, and L. de Repentigny.** 1990. Collaborative evaluation of antigen detection by a commercial latex agglutination test and enzyme immunoassay in the diagnosis of invasive candidiasis. J. Clin. Microbiol. **28:**249–253.
18. **Li, K.-N., D. I. Rouse, and T. L. German.** 1994. PCR primers that allow intergeneric differentiation of *Ascomycetes* and their application to *Verticillium* spp. Appl. Environ. Microbiol. **60:**4324–4331.
19. **Makimura, K., S. Y. Murayama, and H. Yamaguchi.** 1994. Detection of a wide range of medically important fungi by the polymerase chain reaction. J. Med. Microbiol. **40:**358–364.
20. **Makimura, K., S. Y. Murayama, and H. Yamaguchi.** 1994. Specific detection of *Aspergillus* and *Penicillium* species from respiratory specimens by polymerase chain reaction (PCR). Jpn. J. Med. Sci. Biol. **47:**141–156.
21. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
22. **Melchers, W. J. G., P. E. Verweij, P. van den Hurk, A. van Belkum, B. E. de Pauw, J. A. A. Hoogkamp-Korstanje, and J. F. G. M. Meis.** 1994. General primer-mediated PCR for detection of *Aspergillus* species. J. Clin. Microbiol. **32:**1710–1717.
23. **Merz, W. G., and G. D. Roberts.** 1995. Detection and recovery of fungi from clinical specimens, p. 709–722. *In* P. R. Murray, E. J. Baron, M. A. Pfaller, F. C. Tenover, and R. H. Yolken. (ed.), Manual of clinical microbiology, 5th ed. American Society for Microbiology, Washington, D.C.
24. **Odds, F. C.** 1992. Antifungal susceptibility testing of *Candida* spp. by relative growth measurement at single concentrations of antifungal agents. Antimicrob. Agents Chemother. **36:**1727–1737.
25. **Rand, K. H., H. Houck, and M. Wolff.** 1994. Detection of candidemia by polymerase chain reaction. Mol. Cell. Probes **8:**215–222.
26. **Reiss, E., and C. J. Morrison.** 1993. Nonculture methods for diagnosis of disseminated candidiasis. Clin. Microbiol. Rev. **6:**311–323.
27. **Riley, D. E., M. C. Roberts, T. Takayama, and J. N. Krieger.** 1992. Development of a polymerase chain reaction-based diagnosis of *Trichomonas vaginalis*. J. Clin. Microbiol. **30:**465–472.
28. **Saral, R.** 1991. *Candida* and *Aspergillus* infections in immunocompromised patients: an overview. Rev. Infect. Dis. **13:**487–492.
29. **van Deventer, A. J. M., W. H. F. Goessenz, 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.
30. **Verweij, P. E., J. P. Latgé, A. J. M. M. Rijs, W. J. G. Melchers, B. E. de Pauw, J. A. A. Hoogkamp-Korstanje, and J. F. G. M. Meis.** 1995. Comparison of antigen detection and PCR assay using bronchoalveolar lavage fluid for diagnosing invasive pulmonary aspergillosis in patients receiving treatment for hematological malignancies. J. Clin. Microbiol. **33:**3150–3153.
31. **Walsh, T. J., J. W. Hathorn, J. D. Sobel, W. G. Merz, V. Sanchez, S. M. Maret, H. R. Buckley, M. A. Pfaller, R. Schaufele, C. Silva, E. Navarro, J. Lecciones, P. Chandrasekar, J. Lee, and P. A. Pizzo.** 1991. Detection of circulating *Candida* enolase by immunoassay in patients with cancer and invasive candidiasis. N. Engl. J. Med. **324:**1026–1031.
32. **Walsh, T. J., A. Francesconi, M. Kasai, and S. J. Chanock.** 1995. PCR and single-strand conformational polymorphism for recognition of medically important opportunistic fungi. J. Clin. Microbiol. **33:**3216–3220.
33. **Wong, B., and K. L. Brauer.** 1988. Enantioselective measurement of fungal D-arabinitol in the sera of normal adults and patients with candidiasis. J. Clin. Microbiol. **26:**1670–1674.