JOURNAL OF CLINICAL MICROBIOLOGY, Aug. 1994, p. 1902–1907
0095-1137/94/$04.00+0

Vol. 32, No. 8

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

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Received 22 December 1993/Returned for modification 1 March 1994/Accepted 12 May 1994

PCR of a *Candida albicans* cytochrome P-450 lanosterol-α-demethylase (P450-L1A1) gene segment is a rapid and sensitive method of detection in clinical specimens. This enzyme is a target for azole antifungal action. In order to directly detect and identify the clinically most important species of *Candida*, we cloned and sequenced 1.3-kbp fragments of the cytochrome P450-L1A1 genes from *Torulopsis (Candida) glabrata* and from *Candida krusei*. These segments were compared with the published sequences from *C. albicans* and *Candida tropicalis*. Amplimers for gene sequences highly conserved throughout the fungal kingdom were first used; positive PCR results were obtained for *C. albicans*, *T. glabrata*, *C. krusei*, *Candida parapsilosis*, *C. tropicalis*, *Cryptococcus neoformans*, and *Trichosporon beigelii* DNA extracts. Primers were then selected for a highly variable region of the gene, allowing the species-specific detection from purified DNA of *C. albicans*, *T. glabrata*, *C. krusei*, and *C. tropicalis*. The assay sensitivity as tested for *C. albicans* in seeded clinical specimens such as blood, peritoneal fluid, or urine was 10 to 20 cells per 0.1 ml. Compared with results obtained by culture, the sensitivity, specificity, and efficiency of the species-specific nested PCR tested with 80 clinical specimens were 71, 95, and 83% for *C. albicans* and 100, 97, and 98% for *T. glabrata*, respectively.

Systemic candidiasis is an increasing problem, especially in immunocompromised and leukemic patients (17). Diagnosis continues to be based on microscopy and culture. Detection and species identification are often delayed because of the slow growth of *Candida* isolates from clinical specimens. Since pathogenicity and antifungal susceptibility often vary from species to species and since most *Candida* infections are hospital acquired, a rapid and accurate identification to species level would be both clinically and epidemiologically helpful. Several studies seem to indicate that PCR might be a useful test for the early detection of *Candida* species and for their rapid identification to the species level (13, 21, 24). Since publication of our initial report of an assay for the rapid and direct identification of *Candida* species from clinical material by PCR analysis of the cytochrome P-450 lanosterol-α-demethylase (P450-L1A1) gene (2), several studies based on PCR amplification of ribosomal (7, 18), mitochondrial (15, 16), actin (10), or HSP90 (4) genes have been published. The *Candida albicans* L1A1 gene is inhibited by the azole family of antifungal agents and catalyzes an essential step in the conversion of lanosterol (lanosta-8,24-dien-3β-ol) to ergosterol (ergosta-5,7,22-trien-3β-ol), the major membrane sterol specific for fungi (19). In our initial study, we observed additional bands for non-*albicans Candida* species (23), arising most likely from annealing of the first primer pair to ectopic sites, with subsequent spurious amplification.

The purpose of the present study was to design species-specific primers to directly detect and identify the clinically most important species of *Candida*, i.e., *C. albicans*, *Torulopsis (Candida) glabrata*, *Candida tropicalis*, and *Candida krusei*. We therefore isolated and sequenced the L1A1-encoding genes from the genomes of *T. glabrata* and *C. krusei*. Combining direct and nested PCR of conserved and variable L1A1 regions of the four species, it was possible to directly detect and identify these organisms either to the genus or to the species level. The sensitivity and specificity of the assay was tested with artificially seeded specimens as well as with true clinical specimens.

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MATERIALS AND METHODS

Yeast strains and DNA isolation. The seven human pathogenic yeasts analyzed in this study were *C. albicans* ATCC 10231, *T. glabrata* ATCC 2001, *C. krusei* ATCC 749, *C. tropicalis* ATCC 750, *Candida parapsilosis* ATCC 22019, *Cryptococcus neoformans*, and *Trichosporon beigelii*. The last two organisms were isolated from clinical specimens in the Laboratory of Clinical Microbiology of the Centre Hospitalier Universitaire Vaudois in Lausanne, Switzerland. All isolates were maintained on Sabouraud dextrose agar plates. DNA isolation was performed by the following procedure. Yeast cells were grown in YEPD broth (1% yeast extract [Difco], 2% polypeptone [Difco], 2% glucose) and incubated at 30°C overnight, after which the culture was used for DNA preparation. Whole DNA was prepared from spheroplasts as described by Holm et al. (6). The purified DNA was dissolved in TE buffer (10 mM Tris-HCl, 1 mM Na_2-EDTA, pH 8.0).

Negative-control DNA. DNA was extracted from bacterial species as described by Wang et al. (25). One strain per species was used unless indicated otherwise in parentheses. The species were as follows: *Acinetobacter lwoffii*, *Citrobacter freundii*, a *Corynebacterium* sp., an *Enterobacter* sp. (n = 2), *Escherichia coli*, *Gardnerella vaginalis*, *Klebsiella pneumoniae*, a *Micrococcus* sp., *Morganella morganii*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Proteus vulgaris*, *Pseudomonas aerugi-*

nosa, *Salmonella enteritidis*, *Serratia marcescens*, *Shigella sonnei*, a *Staphylococcus* sp. (n = 3), and a *Streptococcus* sp. (n = 3).

Specimen preparation. Seeded specimens tested were blood, urine, or peritoneal fluid. Citrated blood (25) was collected in a 5-ml Monovette (Sarstedt, Nümbrecht, Germany). Seeded samples were prepared by addition of serially diluted amounts of *C. albicans* yeast cell suspension. True clinical specimens tested in this investigation included blood cultures (n = 40), normally sterile body fluids (n = 23) (i.e., deep pus [n = 13], peritoneal fluid [n = 7], pleural fluid [n = 1], cerebrospinal fluid [n = 1], and bile [n = 1]), and other specimens (n = 17) (i.e., urine [n = 12] and bronchoalveolar lavages [n = 5]) and were obtained from 80 patients. All samples were first cultured in the Laboratory of Clinical Microbiology, Centre Hospitalier Universitaire Vaudois, and yeast identification was carried out to the species level by following conventional procedures (14). A 1-ml aliquot of both positive and negative clinical specimens was frozen at −70°C and kept for PCR testing.

An aliquot of the seeded (0.5-ml) or clinical (0.15-ml) specimens was used for DNA preparation. After two washes with washing solution (0.5% Tween 20, 0.5% Triton X-100, 0.5% Nonidet P-40 in water) and one wash with SE (1 M sorbitol, 0.1 M EDTA, pH 8.0), spheroplasts were prepared by treating the cells with 0.5 mg of Zymolyase-100 T per ml and 2% 2-mercaptoethanol in 100 μl of 1 M sorbitol–0.1 M EDTA (pH 8.0) at 37°C for 30 min. After centrifugation at 12,500 × *g* for 2 min, the spheroplasts were washed once with SE buffer, suspended in 100 μl of 1× PCR buffer containing 0.1 mg of proteinase K per ml and 0.1% Nonidet P-40, and incubated at 55°C for 30 min. Samples were then treated for 10 min at 95°C to inactivate the proteinase K, and 1 μl of these samples was used directly for the PCR assay with or without further phenol extraction and ethanol precipitation.

Synthetic oligonucleotides. Oligonucleotides were synthesized with a DNA synthesizer (Applied Biosystems, Inc., Foster City, Calif.). The oligonucleotides used as primers in this study are as follows (see Fig. 2): ATGGGTGGTCAA CATACTTC (DH, bp 1063 to 1082 from *C. albicans*), TA CATCTATGTCTACCACC (1558, bp 1558 to 1541 from *C. albicans*), GTITT(T/C)TA(T/C) TGGATICCITGG (L1A1, bp 298 to 318 from *C. albicans*), CTTCATCAGAAGAGTTA AATG (A, bp 1481 to 1459 from *C. albicans*), TTCGTCAC CACCAGCAGCACT (G, bp 1161 to 1140 from *T. glabrata*), GCATTTTCACCTTCAG (K, bp 1153 to 1133 from *C. krusei*), and GTATCTTCAGAATTGA (T, bp 1421 to 1406 from *C. tropicalis*).

PCR amplification. The PCR was performed with the heat-stable DNA polymerase from *Thermus aquaticus* (*Taq* polymerase; Appligene, Illkirch, France). The final composition of the PCR mix was 0.2 mM (each) deoxynucleotide triphosphates (dATP, dCTP, dGTP, and dTTP), 50 ng of each primer per 50-μl reaction volume, and 1 U of *Taq* polymerase per 50 μl of diluted buffer as provided by the supplier. The PCR was performed in a thermocycler (GeneAmp PCR system 9600; Perkin-Elmer) as follows. The first cycle included 5 min of denaturation at 94°C, 30 s of annealing at the temperature required for the specific primer as described below, and 30 s of primer extension at 72°C followed by 30 cycles of 30 s of denaturation at 94°C, 30 s of annealing at 68°C for primers DH and G, at 55°C for primers DH and 1558 and DH and A, and at 45°C for primers DH and K and DH and T. The annealing step was followed by 30 s of amplification at 72°C. PCR products were stored at −20°C until analyzed.

Detection of amplified PCR products and Southern blot analysis. The PCR products were analyzed by using 0.8% agarose gels and blotted onto a nylon membrane as described by Maniatis et al. (12). Filters were hybridized with 10^6 cpm/ml of random hexanucleotide-primed (^{32}P)dATP-labeled probes. The cDNA fragments used as probes were the *C. albicans* L1A1 fragment, bp +150 to +1410, and the *T. glabrata* fragment, bp +51 to +1445.

Cloning of DNA fragments coding for the cytochrome P450-L1A1 from the genomes of *T. glabrata* and *C. krusei*. The purified DNAs of *T. glabrata* and *C. krusei* were amplified with primers L1A1 and 1558, and the products were subjected to electrophoresis through a low-melting-point 1% agarose gel. A 1,250-bp band was extracted and filled in with Klenow fragment as described by Maniatis et al. (12). Each of the DNA fragments was ligated into the *Sma*I site of the pBluescript SK+ (Stratagene, La Jolla, Calif.) vector, and the ligation mixture was introduced into *E. coli* JM109 by electroporation.

DNA sequencing. Double-stranded DNA subcloned into plasmid pBluescript SK+ was sequenced with a Sequenase version 2.0 sequencing kit (U.S. Biochemicals) according to the supplier's instructions. DNA was annealed with the T3 and T7 primers or synthetic oligonucleotide primers.

RESULTS

Cloning and sequencing of DNA fragments coding for the L1A1 genes of *T. glabrata* and *C. krusei*. The nucleotide sequences of the 1,250-bp cloned fragments from *T. glabrata* and *C. krusei* are shown in Fig. 1, with the 420 deduced amino acid residues. About 50 amino acids are missing at each amino and carboxyl terminus. Comparison with nucleic acid sequences of L1A1 from *C. albicans* (11), *C. tropicalis* (3), and *Saccharomyces cerevisiae* (9) allowed identification of the most extreme 5′ and 3′ highly conserved regions and the design in these regions of the L1A1 and 1558 primers used for the cloning procedure. The localization of these primers is shown in Fig. 2. L1A1 amino acid sequence alignment for *C. albicans*, *C. tropicalis*, *S. cerevisiae*, *T. glabrata*, and *C. krusei* is shown in Fig. 2. Oligonucleotides were designed in order to detect yeasts to the genus and species level, combining amplification from conserved and divergent regions of the L1A1 genes.

Specificity of the PCR. As the DH and 1558 amplimers derive from sequences which are highly conserved (i.e., >90% identical at the amino acid level) in all yeast species studied, we predicted them to be genus rather than species specific. This pair of primers (Fig. 3A) indeed generated amplification products when tested with purified DNA from *C. albicans* (lane 1), *T. glabrata* (lane 2), *C. krusei* (lane 3), and *C. tropicalis* (lane 4). The size of the PCR products obtained with these four *Candida* (*Torulopsis*) species is about 495 bp. Positive PCR products of similar size have also been obtained for *C. parapsilosis* (Fig. 3A, lane 5, weakly positive) and for two other non-*Candida* important pathogenic yeasts, *Cryptococcus neoformans* (lane 6, strongly positive) and *Trichosporon beigelii* (lane 7, positive).

The specificity of this pair of primers was also challenged with a collection of negative-control DNA samples, as detailed in Materials and Methods. When the DH-1558 primer combination was used, the expected 495-bp fragment was generated only for the positive-control DNA, while no amplification was observed for the bacterial or *Aspergillus* DNAs tested.

Four additional primers were derived from the species-specific highly variable regions of the four different cloned genes. Each primer was used in combination with the DH primer, instead of the 1558 primer, in order to selectively amplify *C. albicans*, *T. glabrata*, *C. krusei*, or *C. tropicalis* (Fig. 3B, C, D, and E). Amplimers DH and A amplified a 413-bp product from *C. albicans* DNA (Fig. 3B, lane 1), while primers

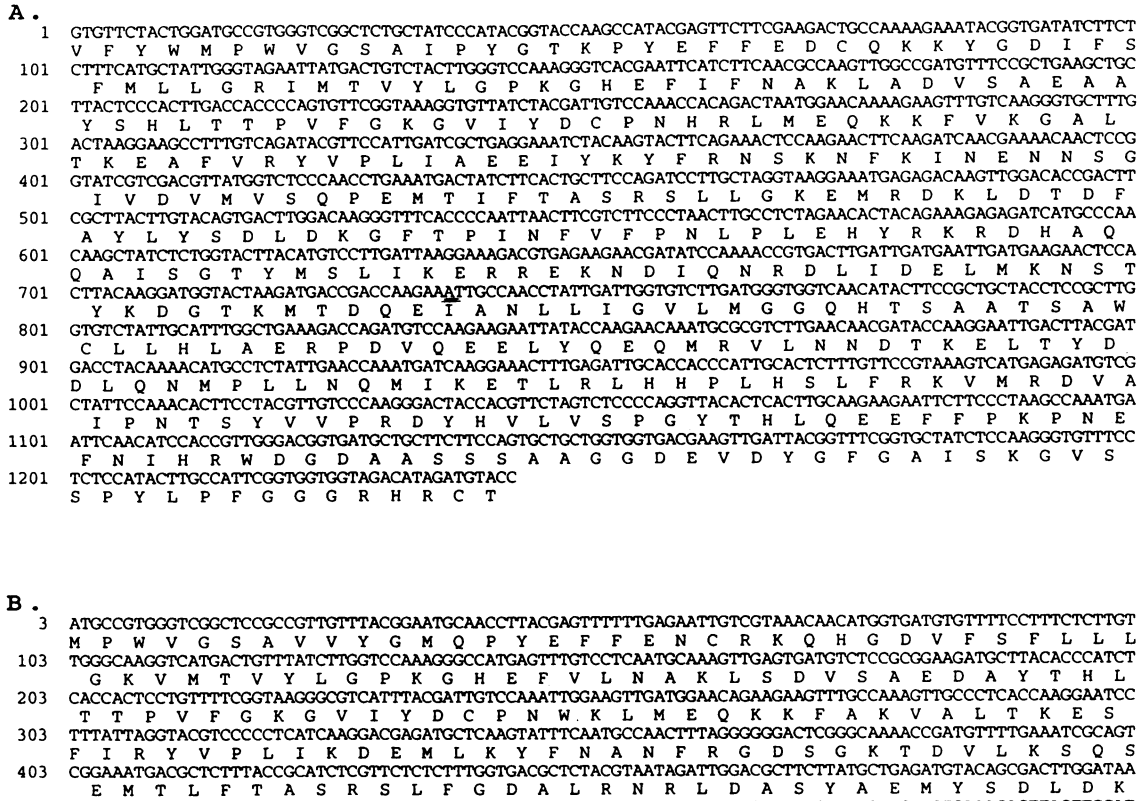

FIG. 1. Sequences of the L1A1 genes from *T. glabrata* (A) and *C. krusei* (B). Nucleotides are numbered on the left, and deduced amino acids are given below the nucleotide sequence. The 1,250 bp of cloned sequence covers about 415 amino acids in the open reading frame.

DH and G amplified a 400-bp product from *T. glabrata* (panel C, lane 2) and a weak product of the same size from *C. parapsilosis* (panel C, lane 5), primers DH and K amplified a 416-bp product from *C. krusei* only (panel D, lane 3), and primers DH and T amplified a 415-bp product from *C. tropicalis* only (panel E, lane 4).

Sensitivity of the PCR for *C. albicans* in seeded blood, urine, or peritoneal fluid. The sensitivity of this PCR assay was tested with seeded clinical specimens containing decreasing amounts of *C. albicans* cells. One microliter of the final DNA preparation was directly amplified, without phenol extraction and ethanol precipitation, in a first PCR using the nonspecific amplimers DH and 1558 (Fig. 4A). One microliter of the first PCR product was then amplified by a nested PCR, using the species-specific primers DH and A (Fig. 4B). PCR products were subjected to a 0.8% agarose gel electrophoresis (Fig. 4, top of each panel) followed by Southern blot hybridization (bottom of each panel) with a *C. albicans* L1A1-specific probe (bp +150 to 1410). Samples were considered PCR positive when the PCR-amplified fragment observed on the agarose gel produced a positive Southern blot hybridization test result.

The detection level for the 495-bp product amplified by direct PCR (Fig. 4A, top) is 10-fold lower than the level for the 413-bp product amplified by nested PCR (panel B, top), and the signal is clearly more intense for all the dilutions with nested PCR. Southern blot analysis with the *C. albicans* L1A1 probe confirmed that PCR products were derived from the *C. albicans* L1A1 gene and increased the detection sensitivity in comparison with that obtained with agarose gel electrophoresis by 1 to 2 orders of magnitude, resulting in a final sensitivity of 10 to 20 cells in seeded blood (Fig. 4), as well as in peritoneal fluid and urine (data not shown). Phenol extraction and ethanol precipitation of the seeded specimens did not further increase the sensitivity.

Species-specific detection of *C. albicans* and *T. glabrata* in clinical specimens. A 150-μl volume of each clinical sample was subjected to the same procedure as that described for seeded samples and tested by PCR. However, the extraction

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          51                                                  92
A         VF YWIPWFGSAA SYGQQPYEFF ESCRQKYGDV FSFMLLGKIM      L1A1
T         VF YWIPWFGSAA SYGMQPYEFF EKCRLKYGDV FSFMLLGKVM
C         VF YWIPWVGSAV VYGMKPYEFF EECQKKYGDI FSFVLLGRVM
G         VF YWMPWVGSAI PYGTKPYEFF EDCQKKYGDI FSFMLLGRIM
K            MPWVGSAV VYGMQPYEFF ENCRKQHGDV FSFLLLGKVM
     93                                                 142
A    TVYLGPKGHE FVFNAKLSDV SAEDAYKHLT TPVFGKGVIY DCPNSRLMEQ
T    TVYLGPKGHE FIYNAKLSDV SAEEAYTHLT TPVFGKGVIY DCPNSRLMEQ
C    TVYLGPKGHE FVFNAKLADV SAEAAYAHLT TPVFGKGVIY DCPNSRLMEQ
G    TVYLGPKGHE FIFNAKLADV SAEAAYSHLT TPVFGKGVIY DCPNHRLMEQ
K    TVYLGPKGHE FVLNAKLSDV SAEDAYTHLT TPVFGKGVIY DCPNWKLMEQ
     143                                                192
A    KKFAKFALTT DSFKRIVPKI REEILNIFVT DESFKLKEKT HGVANVMKTQ
T    KKFAKFALTT DSFKTIVPKI REEVLNIFVN DVSFKTKERD HGVASVMKTQ
C    KKFVKGALTK EAFKSIVPLI AEEVIKIFRD SKNFRLNERT TGTIDVMVTQ
G    KKFVKGALTK EAFVRIVPLI AEEIIKIFRN SKNFKINENN SGIVDVMVSQ
K    KKFAKVALTK ESFIRIVPLI KDEMLKIF..  NANFRG...D SGKTDVLKSQ
     193                                                242
A    PEITIFTASR SLFGDEMRRI FDRSFAQLYS DLDKGFTPIN FVFPNLPLPH
T    PEITIFTASR CLFGDEMRKS FDRSFAQLYA DLDKGFTPIN FVFPNLPLPH
C    PEMTIFTASR SLLGKEMRAK LDTDFAYLYS DLDKGFTPIN FVFPNLPLEH
G    PEMTIFTASR SLLGKEMRDK LDTDFAYLYS DLDKGFTPIN FVFPNLPLEH
K    SEMTLFTASR SLFGDALRNR LDASYAEMYS DLDKGFTPLN FVFSYLPLPN
     243                                                292
A    YWRRDAAQKK ISATYMKEIK LRRERGDIDP NRDLIDSLLI HSTYKDGVKM
T    YWRRDAAQRK ISAHYMKEIK RRRESGDIDP KRDLIDSLLV NSTYKDGVKM
C    YRKRDHAQKA ISGTYMTLIK ERRKNNDIQ. DRDLIDSLMK NSTYKDGVKM
G    YRKRDHAQQA ISGTYMSLIK ERREKNDIQ. NRDLIDELMK NSTYKDGTKM
K    YWKRDAAHKN ISNTYLDLIN TKRAGGEIK. NEDLVDALLK NSVYKDGTRM
     293          DH                                    342
A    TDQEIANLLI GILMGGQHTS ASTSAWFLLH LGEKPHLQDV IYQEVVELLK      DH
T    TDQEIANLLI GVLMGGQHTS ASTSAWFLLH LAEQPQLQDD LYEELTNLLK
C    TDQEIANLLI GVLMGGQHTS AATSAWILLH LAERPDVQQE LYEEQMRVLD
G    TDQEIANLLI GVLMGGQHTS AATSAWCLLH LAERPDVQEE LYQEQMRVLN
K    TDEELAHLMI GVLMGGQHTS SATSAWFLLH LGEKPQLQEE IYREIQSVLG
     343                                                392
A    EKGGDLNDLT YEDLQKLPSV NNTIKETLRM HMPLHSIFRK VTNPLRIPET
T    EKGGDLNDLT YEDLQKLPLV NNTIKETLRM HMPLHSIFRK VMNPLRVPNT
C    .. GGKKELT YDLLQEMPLL NQTIKETLRM HHPLHSLFRK VMKDMHVPNT
G    .. NDTKELT YDDLQNMPLL NQMIKETLRL HHPLHSLFRK VMRDVAIPNT
K    ENFE..RELT YDDLQKLDLV NATIKETLRL HMPLHSIFRK VTRDLPVPNT
     393                                                442
A    NIIVPKGHIV LVSPGYAHTS ERIFDNPEDF DPTRWDTAAA KANSVSF...      A
T    KIVIPKGHIV LVSAGYAHTS DRWFEHPEHF NPRRWESDDT KASAVSF...
C    SIVIPAGTHV LVSPGYTHLR DEIFPNAHQF NIHRWNKDSA .SSY......
G    SIVVPRDTHV LVSPGYTHLQ EEFFPKPNEF NIHRWDGDAA SSSA......      G
K    SIVIPKGHIV LISPGYTMLS ERIFPNASEF QPHRWDEIKS IDGGISFGAE      K
     443                    HR2                         492
A    ..NSSDEVDI GFGKVSKGVS SPYLPFGGGR HRCIGEQFAI VQLGTILTTF      1558
T    ..NSEDTVDI GFGKISKGVS SPYLPFGGGR QRCIGEQFAY VQLGTILTTY      T
C    ..SVGEEVDY GFGAISKGVS SPYLPFGGGR HRCIGEHFAY CQLGVLMSIF
G    ..AGGDEVDI GFGAISKGVS SPYLPFGGGR HRCT
K    GENAKETVDI GFGKISKGVA SPYLPFGGGR HRCT
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FIG. 2. Amino acid sequence alignment for the proteins encoded by the L1A1 genes of *C. albicans* (A), *C. tropicalis* (T), *T. glabrata* (G), *S. cerevisiae* (C), and *C. krusei* (K). The distal helix (DH) and the HR2 domains are delimited by a solid line over the five protein sequences. The location of each primer used in the present study is indicated by underlining below the corresponding protein, and primers are indicated on the right. Gaps in the alignment are indicated by dots.

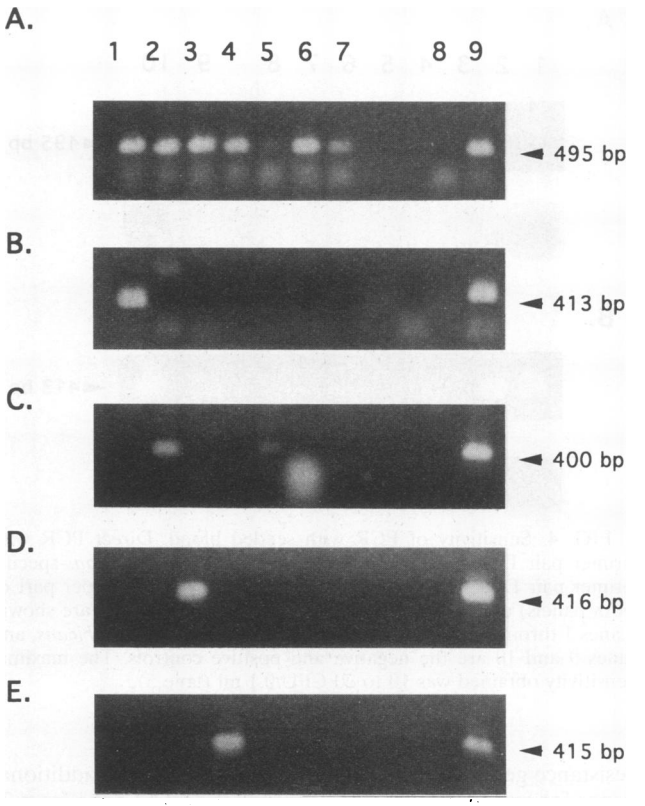

FIG. 3. Specificity of DNA detection by PCR of purified DNA from different yeast strains with ethidium bromide staining on agarose gel electrophoresis. PCR products obtained with the different pairs of primers from different template DNAs were from *C. albicans* (lane 1), *T. glabrata* (lane 2), *C. krusei* (lane 3), *C. tropicalis* (lane 4), *C. parapsilosis* (lane 5), *Cryptococcus neoformans* (lane 6), and *Trichosporon beigelii* (lane 7). Negative and positive controls are shown in lanes 8 and 9. (A) With the DH-1558 primer pair, a 495-bp product is obtained from all yeasts tested. (B) *C. albicans*-specific primer pair DH-A amplifies only *C. albicans*. (C) *T. glabrata*-specific primer pair DH-G amplifies *T. glabrata* (a weak product of the same size is also obtained for *C. parapsilosis*). (D) *C. krusei*-specific primer pair DH-K amplifies only *C. krusei*. (E) *C. tropicalis*-specific primer pair DH-T amplifies only *C. tropicalis*.

step with phenol and ethanol precipitation described above had to be added to decrease the rate of false-negative PCR results attributed to inhibitors, especially in specimens other than blood cultures. This step also prevented DNA degradation and allowed repeated amplification from the same DNA preparation. After the purification step, the 80 clinical samples were first amplified with the primer pair DH-1558 and then with the *C. albicans* (DH-A)- or *T. glabrata* (DH-G)-specific primers in a nested PCR. From the 41 *C. albicans* or *T. glabrata* culture-positive samples, 19 gave a positive signal with the first PCR. After the nested-PCR round, 27 of 38 *C. albicans* and 7 of 7 *T. glabrata* culture-positive specimens gave a positive signal (Table 1). This corresponds to an overall sensitivity of 76% for both species (71% for *C. albicans* and 100% for *T. glabrata*). The specificity was 95% for *C. albicans*, with 37 of the 39 *C. albicans* culture-negative specimens nested PCR negative (Table 1), and 97% for *T. glabrata*, with 71 of the 73 *T. glabrata* culture-negative specimens nested PCR negative. One blood culture specimen which was culture positive for both species gave a nested-PCR signal with both pairs of primers, DH-A and DH-G. The 13 specimens which were culture positive for yeasts other than *C. albicans* or *T. glabrata* (Table 1) were PCR negative when amplified in the second nested-PCR round with the DH-A and DH-G primers, although 1 blood culture containing *Candida kefyr* produced a signal when amplified in the first PCR round with the DH-1558 primer pair. Thus, the efficiency of the nested PCR was 80% for *C. albicans* and 98% for *T. glabrata*.

DISCUSSION

We developed a PCR assay to simultaneously detect and identify the clinically most important species of *Candida* directly by species-specific amplification of the L1A1 gene. Regarding the choice of the most appropriate target DNA and amplification strategy, various probes have been proposed for the diagnosis of candidiasis (4, 7, 10, 15, 16, 18). Most authors selected ribosomal genes as target DNA for PCR amplification, expecting an increased sensitivity with a multiple-copy-gene target DNA. Our strategy to use the cytochrome P450-L1A1 as a target probe was based on the fact that this enzyme is specific for fungi (19) and is inhibited by the azole family of antifungal drugs. Resistance to azole antifungal agents related to sequence alteration of the L1A1 gene might thus be detected in the future by PCR, as shown for the tetracycline

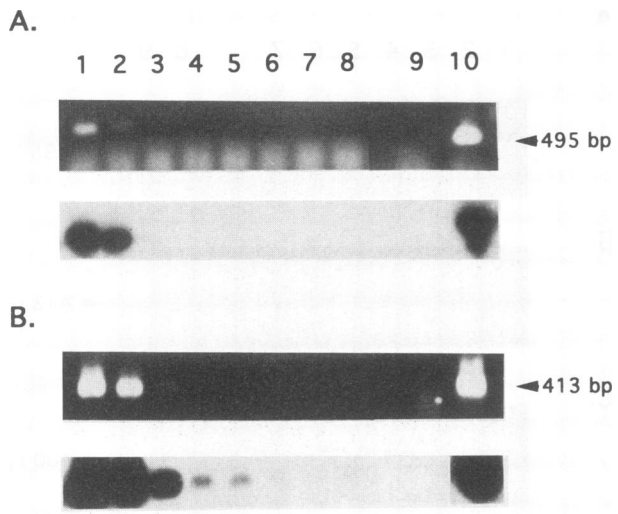

FIG. 4. Sensitivity of PCR with seeded blood. Direct PCR with primer pair DH-1558 (A) and nested PCR with *C. albicans*-specific primer pair DH-A (B) by agarose gel electrophoresis (upper part of both panels) or Southern blot (lower part of both panels) are shown. Lanes 1 through 8 contain serially diluted amounts of *C. albicans*, and lanes 9 and 10 are the negative and positive controls. The maximal sensitivity obtained was 10 to 20 CFU/0.1 ml (lane 5).

resistance gene (22). We cloned and sequenced two additional genes for enzymes of the P-450 family: the L1A1 gene from *T. glabrata* and that from *C. krusei*. They were found to be highly similar to the L1A1 genes from *C. albicans* (11), *C. tropicalis* (3), and *S. cerevisiae* (9). A higher degree of identity (at the protein level) was observed between *T. glabrata* and *S. cerevisiae* (90%) than between *C. albicans* and *C. tropicalis* (80%), while *C. krusei* seems as distantly related to *C. albicans* as to *C. tropicalis* or *S. cerevisiae* (80%). Sequence comparison of the different members of the cytochrome P-450 family indicates that regions with a high degree of similarity within the carboxy-terminal portions are likely to be correlated with common functions such as heme binding and interaction with membrane-bound reductase to express the substrate-specific catalytic activity (1). The putative heme-binding domain, i.e., the HR2 region, resides in the carboxy-terminal portion (5). A high degree of sequence similarity in the active site of the enzyme is expected. Thus, a hydrophobic stretch (defined as the distal helix) is highly conserved in all P-450s (20). Amino acid substitution within this region causes a loss of enzyme activity (8). The first PCR, with the genus-specific primer set DH-1558, was designed to amplify the conserved distal helix and the HR2 regions. It detected 46% of the *C. albicans* and *T. glabrata* culture-positive specimens. The second nested PCR was done with the species-specific primer set DH-X, where oligonucleotides X have been selected in a sequence encoding a highly divergent segment of the carboxy-terminal region of the protein. It detected 76% of the *C. albicans* or *T. glabrata* culture-positive specimens. The sensitivity for *C. albicans* was raised to 86% in the subset of blood culture samples. The seven *T. glabrata* culture-positive specimens were all detected. Eleven specimens were PCR negative while culture positive, and four were PCR positive while culture negative. Nine false-negative PCR results were obtained for *C. albicans* in body fluids, while only two were observed with blood culture specimens. Among the PCR false-negative samples, two were thick pus and required special procedures such as heating and prolonged vortexing before they could even be pipetted. Conventional culture results for five of these PCR false-negative samples indicated a low concentration of *Candida* cells. There were two false-positive PCR results for each species. Those for *C. albicans* occurred with nonsterile body fluids, i.e., one urine specimen and one bronchoalveolar lavage specimen, from a human immunodeficiency virus-positive patient with documented oral candidosis. These reproducibly PCR-positive results are likely to be due to sample contamination with the patient's endogenous flora. Although these specimens were included in the series to broaden the spectrum of clinical samples tested, they are clinically irrelevant because these sites are frequently colonized by yeasts. In this report, we have compared biochemical (PCR) versus biological amplification of yeasts in seeded and true specimens with respect to sensitivity, specificity, and speed. While PCR is somewhat less sensitive than culture techniques, the increment in speed of

TABLE 1. Nested PCR versus culture for detection of yeasts

Clinical specimens (n = 80)	No. culture positive for:			No. culture negative for all yeasts	No. PCR positive for:		No. PCR false	
	C. albicans	*T. glabrata*	Other yeasts		*C. albicans*	*T. glabrata*	Negative	Positive
Blood cultures (n = 40)	16[a]	5[a]	10[b]	10	14	5	2	2[c]
Normally sterile body fluids[d] (n = 23)	17	1	2[e]	3	11	1	6	
Other[f] (n = 17)	5	1	1[g]	10	2	1	3	2[h]
Total	38[a]	7[a]	13	23	27	7	11	4

[a] One of these samples contains *C. albicans* and *T. glabrata*.
[b] *Candida guilliermondii* (n = 1), *C. kefyr* (n = 1), *C. parapsilosis* (n = 4), *Candida famata* plus *C. guilliermondii* (n = 1), and *C. neoformans* (n = 3).
[c] With the DH-G (*T. glabrata*) primer pair.
[d] Deep pus (n = 13), peritoneal fluid (n = 7), pleural fluid (n = 1), cerebrospinal fluid (n = 1), and bile (n = 1).
[e] *C. tropicalis* (n = 1) and *C. krusei* (n = 1).
[f] Bronchoalveolar lavages (n = 5) and urine (n = 12).
[g] *C. tropicalis* (n = 1).
[h] With the DH-A (*C. albicans*) primer pair.

diagnosis and species identification may well be clinically important. The decrement in sensitivity of the PCR technique can be attributed to two major causes. First, when the specimen contains a limiting amount of *Candida* cells, the mere difference in sample size can lead to discrepant results according to the Poisson distribution law. Second, clinical specimens, in comparison with seeded specimens, contain a significantly greater amount of leukocytes, cellular debris, or bacteria as potential sources of strong inhibitors. Our results compare well with the only other similar clinical study, published by Crampin and Matthews (4), concerning the species-specific identification of a single species, i.e., *C. albicans*. From their data, it is possible to calculate a sensitivity of 81% and a specificity of 83% for the clinical specimens analyzed. The majority of the samples included in that study originated from normally nonsterile body sites, and the few blood specimens tested were seeded blood specimens. A cross-reactivity of the primers with non-*albicans Candida* species was mentioned by the authors but was not investigated further. Our method has been validated with true clinical specimens for the species-specific identification of two species, *C. albicans* and *T. glabrata*, which represent more than 90% of all *Candida* infections at the Centre Hospitalier Universitaire Vaudois. Their rapid identification is clinically relevant because of the marked difference in the antifungal resistance patterns of these two species. Because of their rare occurrence in clinical specimens, species-specific detection of *C. krusei* and *C. tropicalis* could only be tested with purified template DNA. However, these two species might be detected similarly with clinical specimens, by using primers selected by comparing the L1A1 sequences published for *C. tropicalis* and *C. albicans* with the newly cloned sequences of *T. glabrata* and *C. krusei*. Likewise, other species-specific primers deduced from the L1A1 sequences of additional species or primers derived from alternate conserved regions of the gene could be used in a nested PCR to identify yeasts to the genus or to the species level.

ACKNOWLEDGMENTS

We thank M. Monod and K. Jaton-Ogay for their gift of *Aspergillus fumigatus*, *Mycobacterium tuberculosis*, and *Listeria monocytogenes* DNA and C. Durussel for preparing the clinical specimens.

This work was supported by the Swiss National Fund, the Roche Foundation, and by a research fellowship awarded to T.G.B. by the American College of Surgeons.

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