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Detection and Identification of Fungal Pathogens in Blood by Using Molecular Probes

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A PCR assay was developed for the detection and identification of *Candida* and *Aspergillus* species. The design of the oligonucleotide primer pair as well as the species-specific probes used for species identification was derived from a comparison of the sequences of the 18S rRNA genes of various fungal pathogens. The primers targeted a consensus sequence for a variety of fungal pathogens. The assay was tested for sensitivity and specificity with 134 fungal and 85 nonfungal isolates. To assess clinical applicability, 601 blood samples from four defined groups were tested: group A ($n = 35$), controls; groups B to D ($n = 86$), patients with febrile neutropenia, without fungal colonization (group B; $n = 29$) and with fungal colonization (group C; $n = 36$); and patients with documented invasive fungal infection (IFI) (group D; $n = 21$). The assay detected and, by species-specific hybridization, identified most of the clinically relevant *Candida* and *Aspergillus* species at 1 CFU/ml of blood. Amplification was 100% sensitive for all molds and yeasts tested, with *Histoplasma capsulatum* being the only non-*Aspergillus* species hybridizing with the *Aspergillus* spp. probe. None of 35 group A patients and only 3 of 65 group B and C patients were PCR positive. The sensitivity of the assay for specimens from patients with IFI (21 patients in group D) was 100% if two specimens were tested. For specificity, 3 of 189 specimens from patients at risk but with negative cultures were positive by the assay, for a specificity of 98%. PCR preceded radiological signs by a median of 4 days (range, 4 to 7 days) for 12 of 17 patients with hepatosplenic candidiasis or pulmonary aspergillosis. For the 10 patients with IFI responding to antifungal therapy, PCR assays became persistently negative after 14 days of treatment, in contrast to the case for 11 patients, who remained PCR positive while not responding to antifungal therapy. Thus, the described PCR assay allows for the highly sensitive and specific detection and identification of fungal pathogens in vitro and in vivo. Preliminary data from the screening of a selected group of patients revealed some value in the early diagnosis and monitoring of antifungal therapy.

In recent years, yeasts and molds have emerged as important nosocomial pathogens (1, 13, 23, 29, 35). These fungi are a leading cause of morbidity and mortality in cancer (13, 36), burn, and surgical patients as well as neonatal intensive care unit patients (17).

Early initiation of antifungal therapy is critical in reducing the high mortality rate in these patients (9, 21). Despite intensive efforts by many investigators, early and rapid diagnosis of systemic fungal infections remains limited. Culture detection of fungal species is often delayed because of slow or absent growth of fungal isolates from clinical specimens. Blood cultures are positive for fewer than 50% of patients with hepatosplenic candidiasis (2, 33) and are rarely positive for patients with invasive aspergillosis (7).

Thus, rapid and more sensitive diagnostic strategies for fungal infection including the detection of antigen or DNA are being evaluated (3–6, 10–12, 15, 16, 19, 20, 22, 24, 25, 27, 28, 31, 33, 35). PCR-based detection methods, primarily with primers targeted to gene sequences unique to fungi (4, 16), have been used. Multicopy gene targets for increasing the sensitivity of detection of fungal infection (22, 27, 32) and universal fungal PCR primers for broadening the range of detectable fungi (10, 11, 15, 19, 24, 35) have been evaluated. Difficulties with these assays were found to be coamplification of human DNA (16) or lack of adaptation to human specimens (6). We developed a PCR assay that amplified a highly conserved sequence of the multicopy 18S rRNA gene and analyzed its sensitivity and specificity and the range of fungal pathogens detectable by the assay. Furthermore, to assess its clinical applicability, a large number of blood samples from three groups of clearly defined patients as well as from healthy control persons were simultaneously analyzed by PCR and culture assay.

MATERIALS AND METHODS

Organisms and growth conditions. The following stock fungal organisms, yeasts, and molds were obtained from the German Collection of Microorganisms (DSM); the Centraal Bureau voor Schimmelcultures (CBS), Baarn, The Netherlands; and the American Type Culture Collection (ATCC), Rockville, Md., or the indicated departments of clinical microbiology: *Candida albicans* DSM 6569, *Torulopsis glabrata* DSM 6425, *Candida krusei* DSM 3433, *Candida tropicalis* DSM 5991, *Candida parapsilosis* DSM 5784, *Candida guillermondii* ATCC 6260, *Trichosporon cutaneum* DSM 70675, *Aspergillus fumigatus* DSM 790, *Aspergillus flavus* DSM 1959, *Aspergillus versicolor* DSM 1943, *Aspergillus niger* DSM 1957, *Aspergillus nidulans* DSM 3365, *Aspergillus terreus* DSM 1958, *Malassezia furfur* DSM 6170, *Fusarium* spp. (clinical isolates from the Fred Hutchinson Cancer Research Center [FHCRC]), *Trichosporon cutaneum* CBS 2466, *Mucor racemosus* (clinical isolates from FHCRC), *Penicillium* spp. (clinical isolates from FHCRC), *Pseudallescheria boydii* (clinical isolates from FHCRC and Department of Medical Microbiology, University of Tübingen), *Paecilomyces variotii* (clinical isolates from FHCRC), *Histoplasma capsulatum* (obtained from CBS), and *Saccharomyces cerevisiae* CBS 1171 (and clinical isolates from the Depart-

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ment of Medical Microbiology, University of Tübingen) (see Tables 1 and 2). Clinical isolates of the fungal pathogens listed above and tested in addition to the reference strains were identified as described below and were obtained from the Department of Medical Microbiology in Tübingen and FHCRC. In addition, a panel of microorganisms, identified by standard methods, was chosen to represent bacteria and viruses, including *Clostridium perfringens*, *Bacteroides fragilis*, *Bacillus cereus*, *Pseudomonas aeruginosa*, *Nocardia asteroides*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus mitis*, *Streptococcus sanguis*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Escherichia coli*, *Borrelia burgdorferi*, *Propionibacterium acnes*, Epstein-Barr virus (EBV), cytomegalovirus (CMV), herpes simplex virus (HSV) types 1 and 2, human herpesvirus 6 (HHV6), and parvovirus B19 (see Table 2). To assess amplification and hybridization of human DNA specimens, DNA extracted from human fibroblasts and lung and liver biopsy specimens was also tested in the assay (see Table 2).

Clinical cultures and identification of fungal pathogens. Blood samples were cultured by bedside inoculation into BACTEC fungal medium (Becton Dickinson, Heidelberg, Germany) and were tested daily for microbial growth by infrared detection (BACTEC 860; Becton Dickinson). When positive, samples were subcultured onto Sabouraud medium for identification. Identification of yeasts (except *M. furfur*) was performed by the germ tube test in sterile bovine serum, by morphology on rice agar, and by testing 19 assimilation reactions with the API 20 aux system (API Biomérieux, Nürtingen, Germany). *M. furfur* was identified by morphology, urease activity was tested with a Vitek yeast card (API Biomérieux) and lipid-dependent growth was tested by parallel subculturing on Sabouraud agar and lipid-containing yeast medium. Molds were differentiated by microscopic examination (lactopherol cotton blue tape mounts and slide cultures), colony morphology, and testing for growth at various temperatures.

DNA extraction from fungal suspensions. Fungal isolates were cultured on Sabouraud medium at 30°C; *Candida* isolates were cultured for 48 h, and *Aspergillus* isolates were cultured for 72 h. Thereafter, fungal saline suspensions were adjusted photometrically (A_{530}; McFarland no. 0.5 standard) to a concentration of 1×10^6 to 5×10^6 cells/ml. Tenfold serial dilutions (10^6 to 10^0 cells) were prepared to test the sensitivity and specificity of the assay. The fungal suspensions with predetermined numbers of CFU per milliliter were centrifuged at $5{,}000 \times g$, and then the pellet was incubated with 5 ml of leukocyte lysis buffer (WCLB) consisting of 10 mM Tris (pH 7.6), 10 mM EDTA (pH 8.0), 50 mM NaCl, 0.2% sodium dodecyl sulfate (SDS), and 200 μg of proteinase K (Boehringer Mannheim, Mannheim, Germany) per ml at 65°C for 2 h. Zymolyase, a 1-3-glucan laminaripentaohydrolase, may not be solely used for spheroplasting for many fungi due to their cell wall composition (especially *A. niger*, *A. terreus*, and *T. cutaneum*). Thus, samples were then pelleted and incubated with 50 mM NaOH at 95°C for 10 min. Neutralization with 1 M Tris (pH 7.0) was followed by treatment with 300 μg of Zymolyase (ICN, Costa Mesa, Calif.) per ml in 50 mM Tris (pH 7.5), 10 mM EDTA, and 28 mM β-mercaptoethanol at 37°C for 45 min to give plasts. If the solution was nonhomogeneous after this step, the concentration of Zymolyase was increased or the solution was sonicated for 20 min. After centrifugation at $5{,}000 \times g$, pellets were treated with 1 M Tris-EDTA and 10% SDS at 65°C for 30 min for plast lysis. Then, 5 M potassium acetate was added and the samples were incubated at −20°C for 30 min for protein precipitation. After an additional centrifugation step at $1{,}000 \times g$ for 20 min, DNA precipitation was carried out with cold isopropanol. DNA was purified with 70% ethanol, and the DNA was air dried and resuspended in 40 μl of double-distilled H_2O. DNA content and purity were determined by spectral photometry at 260, 280, and 320 nm. Samples were then diluted to a final concentration of 50 ng/μl.

Moreover, cell suspensions were spiked into uninfected EDTA-anticoagulated blood from healthy individuals, and DNA was extracted as described below.

DNA extraction from blood specimens. After hypotonic lysis of the erythrocytes with RCLB (10 mM Tris [pH 7.6], 5 mM $MgCl_2$, 10 mM NaCl), followed by enzymatic lysis of the leukocytes with WCLB (10 mM Tris [pH 7.6], 10 mM EDTA, 50 mM NaCl, 0.2% SDS, 200 μg of proteinase K per ml) at 65°C for 45 min, the samples were pelleted and incubated with 50 mM NaOH at 95°C for 10 min. Neutralization with 1 M Tris (pH 7.0) was followed by treatment with 300 μg of Zymolyase (ICN) per ml in 50 mM Tris (pH 7.5), 10 mM EDTA, and 28 mM β-mercaptoethanol at 37°C for 45 min to give plasts. After centrifugation at $5{,}000 \times g$ the supernatant containing human DNA and proteins was decanted, and the pellets were treated with 1 M Tris-EDTA and 10% SDS at 65°C for 30 min for plast lysis. Then, 5 M potassium acetate was added and the samples were incubated at −20°C for 30 min for protein precipitation. After an additional centrifugation step at $1{,}000 \times g$ for 20 min, DNA precipitation from the supernatant was carried out with cold isopropanol. DNA was purified with 70% ethanol, air dried, and resuspended in 40 μl of H_2O. After spectral photometry, the samples were then diluted to a final concentration of 50 ng/μl. DNA extraction from bacteria, viruses, and tissue was performed as described previously (8).

PCR assay. (i) Oligonucleotide design. The designs of the oligonucleotide primer pair (5′-ATTGGAGGGCAAGTCTGGTG and 5′-CCGATCCCTAGTCGGCATAG) and the DNA probes for *C. albicans* (TCTGGGTAGCCATTTATGGCGAACCAGGAC), *T. glabrata* (TTCTGGCTAACCCCAAGTCCTTGTGGCTTG), *C. krusei* (GTCTTTCCTTCTGGCTAGCCTCGGGCGAAC), *C. tropicalis* (GTTGGCCGGTCCATCTTTCTGATGCGTACT), and *C. parapsilosis* (TTTCCTTCTGGCTAGCCTTTTTGGCGAACC), as well as the *Aspergillus* DNA probe (CATGGCCTTCACTGGCTGTGGGGGGAACCA) and the DNA probe specifically hybridizing with *A. fumigatus*, *A. flavus*, and *A. versicolor* (TGGGGAACCTCATGGCCTTCACTGGCTGTG) used in this study, were derived from a comparison of the sequences of 18S rRNA genes (ribosomal DNA) in the GenBank database (EMBL and DDBJ databases). The primers target a consensus sequence for a variety of fungal pathogens. Amplification with the primers described above yields a 482- to 503-bp fragment, depending on the fungal pathogen tested. The binding sites of the primers in *C. albicans* DNA are from positions 544 to 563 and 1033 to 1014, respectively. Probes bind within the variable areas of the gene, V7 to V9.

(ii) Amplification. Amplification reactions were performed in a 50-μl volume (containing 10 mM Tris [pH 9.6], 50 mM NaCl, 10 mM $MgCl_2$, 200 μg of bovine serum albumin per ml, 0.5 mmol of deoxyribonucleotide triphosphates per liter, 100 pmol of forward and reverse primers, and 1.5 U of *Taq* polymerase [Amersham, Braunschweig, Germany]). Extracted DNA (100 ng) was added, and 35 cycles of repeated denaturation, primer annealing, and enzymatic chain extension were performed in a Biomed thermocycler (model 60). The amplification program was carried out as follows: 30 s at 94°C, 1 min at 62°C, and 2 min at 72°C, followed by a single terminal extension at 72°C for 5 min. To monitor for contamination, aliquots of saline and human fibroblast DNA were prepared concurrently as negative extraction and amplification controls and were tested by the same procedure described above. Each sample set amplified contained 10 to 20% nonfungal DNA samples.

To minimize the risk of contamination, the recommendations of Kwok and Higuchi (18) were followed. Furthermore, the area where the PCR technique was performed was physically separated from the laboratory area where DNA extraction and recombinant DNA research were performed. To exclude the presence of polymerase inhibitors and to control the quality of the extracted DNA, a fragment of the human HLA class I gene (4th exon, 129 bp long) (8) was amplified in all samples in parallel.

(iii) Detection of the amplification products. Ten-microliter aliquots of each amplification product were electrophoretically separated in a 2% agarose gel in 1× TAE buffer (pH 8.0; 40 mM Tris-acetate [pH 7.5], 2 mM sodium EDTA), followed by ethidium bromide staining. The amplicon was transferred by vacuum filtration onto nylon membranes (Hybond; Amersham, Braunschweig, Germany) for slot or Southern blotting, followed by alkaline DNA denaturation by incubating the membrane in 0.4 N NaOH for 20 min. Internal oligonucleotide probes specific for the fungal pathogens (see above) were synthesized and 5′ labeled with digoxigenin by using the terminal transferase kit (Boehringer Mannheim) with 30 pM oligonucleotide and 1× reaction buffer (2.5 mM $CoCl_2$, 25 U of terminal transferase, 0.1 mM digoxigenin-dUTP). Each of the PCR products was hybridized with 30 pM labeled oligonucleotide for 20 min at 42°C. The salt (0.75 M NaCl, 0.15 M $NaH_2PO_4 \cdot H_2O$) and detergent (1% SDS) contents of the washing solutions were adapted for a stringent washing procedure. Washing temperatures close to the melting point of the oligonucleotides were chosen, and the membranes were then washed twice for 7 min at 76°C. Hybrids were then incubated with anti-digoxigenin-alkaline phosphatase (Fab fragments of 150 mU/ml; Boehringer Mannheim) for 20 min and thereafter were visualized with nitroblue tetrazolium (75 mg/ml in dimethylformamide) and bromochlorindoyl phosphate toluidine (Boehringer Mannheim) (50 mg/ml in dimethylformamide) for 10 min up to 2 h.

Sensitivity studies. To determine the sensitivity and the limit of detection, PCR assays were performed with 10 pg, 1 pg, 100 fg, 50 fg, and 10 fg of genomic DNA of *C. albicans*, *C. tropicalis*, *C. krusei*, *C. parapsilosis*, *T. glabrata*, *A. fumigatus*, *A. terreus*, *A. niger*, *A. flavus*, *A. versicolor*, and *A. nidulans*. To determine the sensitivity of the assay for the detection of fungal pathogens in blood, suspensions of all 11 fungal pathogens listed above were diluted (1:10, 1:100, . . . $1:10^6$) in sterile saline. One milliliter of each dilution step was added to 9 ml of blood, and the mixture was inoculated into 30 ml of BACTEC fungal medium. Then, 10 ml of the inoculated blood culture medium was removed for DNA extraction and amplification. A total of 40 μl of each dilution step was also transfered to a Sabouraud agar plate for colony counting (determination of numbers of CFU).

Patients. Between January 1992 and December 1994 selected groups of patients with febrile neutropenia (<500 neutrophils/μl with a temperature of >38.5°C) were screened for fungal infection by blood culture and PCR assay simultaneously at Tübingen University Hospital. Thirty-five controls and 86 patients who had undergone bone marrow transplantation ($n = 33$) or remission induction therapy ($n = 53$) for hematological malignancies ($n = 48$) and solid tumors ($n = 5$) met the eligibility criteria and were placed in the following groups: Group A ($n = 35$) consisted of healthy controls. Group B ($n = 29$) consisted of patients without documented colonization (no fungal pathogens were isolated from oropharygeal, rectal, vaginal or catheter entrance, or stool specimens) and no evidence of invasive fungal infection (IFI; blood culture negative, no pulmonary infiltrates, no neurological symptoms, and improvement without antifungal therapy). Group C ($n = 36$) consisted of patients with documented colonization by fungal pathogens but no evidence of invasive fungal disease. Group D ($n = 21$) consisted of patients with documented IFI, which was defined as satisfying at least one of the following criteria: (i) a tissue biopsy specimen from a lesion clinically documented to be positive for a fungal pathogen by histology and culture ($n = 12$); (ii) the combination of the isolation of *Aspergillus* species from bronchoalveolar lavage fluid from a neutropenic patient with new pulmonary infiltrates exhibiting characteristic features such as the halo

TABLE 1. *Candida* and *Aspergillus* species and strains tested by the designed PCR assay

Fungal species	No. positive/total no. of reference strains and clinical isolates tested by PCR							
	Amplification positive	Positive by hybridization with DNA-probe specific for:						
		C. albicans	*C. tropicalis*	*C. parapsilosis*	*T. glabrata*	*C. krusei*	*Aspergillus* spp.	*A. fumigatus*[a]
C. albicans	40/40	40/40	0/40	0/40	0/40	0/40	0/40	0/40
C. tropicalis	10/10	0/10	10/10	0/10	10/10	0/10	0/10	0/10
C. parapsilosis	6/6	0/6	0/6	6/6	0/6	6/6	0/6	0/6
T. glabrata	11/11	0/11	0/11	0/11	11/11	0/11	0/6	0/6
C. krusei	8/8	0/8	0/8	0/8	0/8	8/8	0/6	0/6
C. guillermondii	2/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2
C. kefyr	3/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
A. fumigatus	8/8	0/8	0/8	0/8	0/8	0/8	8/8	8/8
A. flavus	6/6	0/6	0/6	0/6	0/6	0/6	6/6	6/6
A. terreus	5/5	0/5	0/5	0/5	0/5	0/5	5/5	5/5
A. niger	7/7	0/7	0/7	0/7	0/7	0/7	7/7	0/7
A. nidulans	5/5	0/5	0/5	0/5	0/5	0/5	5/5	0/5
A. versicolor	3/3	0/3	0/3	0/3	0/3	0/3	3/3	0/3

[a] This DNA probe hybridizes with *A. fumigatus*, *A. flavus*, and *A. versicolor.*

or crescent sign and pleural-based angulated lesions on computed tomography scan ($n = 5$); or (iii) at least one blood sample from a febrile patient culture positive for *Candida* spp. (candidemia) ($n = 4$).

Study design. According to the criteria presented above, patients were retrospectively assigned to groups A to D. Thus, all patients receiving empirical antifungal therapy but not fulfilling the criteria for an invasive fungal infection as defined above were excluded from the analyses.

Blood samples were drawn twice prior to and three times per week during antifungal therapy. Blood samples were divided for assay by PCR and culture. Testing was performed by an investigator blinded to the clinical and culture data. The clinicians following the patients were blinded to the PCR data, which were not used in the diagnosis or for patient management. Data analysis was performed after completion of the clinical and testing phases of the study.

Nucleotide sequence accession numbers. The numbers were as follows: GenBank database (EMBL and DDBJ databases) *C. albicans*, M60302; *C. tropicalis*, M60308; *C. krusei*, M60305; *C. parapsilosis*, M60307; *T. glabrata*, M60311; *A. fumigatus*, M60300; *A. terreus*, X78540; *A. niger*, X78537; *A. flavus*, X78537; and *A. nidulans*, X78539.

RESULTS

PCR methodology. (i) Range of detectable fungal pathogens. All clinical isolates and reference strains of *C. albicans*, *T. glabrata*, *C. tropicalis*, *C. parapsilosis*, *C. krusei*, *A. fumigatus*, *A. flavus*, *A. terreus*, *A. niger*, *A. nidulans*, and *A. versicolor* tested were successfully amplified and detected by ethidium bromide-stained gel analysis and hybridization with the species-specific or genus-specific oligonucleotide probes (Table 1).

Additional fungal pathogens (*M. furfur*, *Fusarium* spp., *T. cutaneum*, *Mucor* spp., *Penicillium* spp., *P. boydii*, *P. variotii*, *H. capsulatum*, and *S. cerevisiae*) for which species-specific DNA probes have not been designed were also successfully amplified with the defined primer pair (Table 2). All clinical isolates and laboratory strains were included in the study prior to knowing the result of the PCR assay.

(ii) Sensitivity. By using ethidium bromide staining, in four different experiments 100 fg of DNA of two strains of each of the following species tested was detectable: *C. albicans*, *T. glabrata*, *C. tropicalis*, *C. parapsilosis*, *A. fumigatus*, *A. flavus*, *A. versicolor*, *A. terreus*, *A. niger*, and *A. nidulans*. The two strains of *C. krusei* were detected in the agarose gel at a sensitivity level of only 1 pg. By Southern blot hybridization the sensitivity was increased to 10 fg of DNA for the two strains of *C. albicans* (Fig. 1) and to 50 fg of DNA for all other fungal isolates tested. When culturing these yeast and mold cells in blood, this correlated with 1 CFU/ml of blood, as demonstrated in five different experiments with two strains of each of the 11 fungal pathogens listed above.

This sensitivity was achieved only for the *Aspergillus* species tested by using an alkaline-heat treatment of the samples. The Zymolyase digestion step alone did not release sufficient DNA from *Aspergillus conidia* to allow for sensitive detection.

TABLE 2. Additional fungal and nonfungal pathogens tested by the designed PCR assay

Fungal and nonfungal pathogens	No. positive/total no. of reference strains and clinical isolates tested by PCR			
	Amplification positive	Positive by hybridization with DNA probe specific for:		
		Candida spp. (five DNA probes)[a]	*Aspergillus* spp.	*A. fumigatus*[b]
Malassezia furfur	3/3	0/3	0/3	0/3
Fusarium spp.	3/3	0/3	0/3	0/3
Trichosporon cutaneum	2/2	0/2	0/2	0/2
Mucor spp.	3/3	0/3	0/3	0/3
Penicillium spp.	2/2	0/2	0/2	0/2
Pseudallescheria boydii	1/1	0/1	0/1	0/1
Paecilomyces spp.	2/2	0/2	0/2	0/2
Histoplasma capsulatum	2/2	0/2	2/2	0/2
Saccharomyces cerevisiae	2/2	0/2	0/2	0/2
Clostridium perfringens	0/3	0/3	0/3	0/3
Bacteroides fragilis	0/2	0/2	0/2	0/2
Bacillus cereus	0/3	0/3	0/3	0/3
Pseudomonas aeruginosa	0/4	0/4	0/4	0/4
Nocardia asteroides	0/2	0/2	0/2	0/2
Listeria monocytogenes	0/3	0/3	0/3	0/3
Staphylococcus aureus	0/5	0/5	0/5	0/3
Staphylococcus epidermidis	0/6	0/6	0/6	0/6
Streptococcus mitis	0/5	0/5	0/5	0/5
Streptococcus sanguis	0/3	0/3	0/3	0/3
Klebsiella pneumoniae	0/4	0/4	0/4	0/4
Enterobacter cloacae	0/6	0/6	0/6	0/6
Escherichia coli	0/5	0/5	0/5	0/5
Borrelia burgdorferi	0/3	0/3	0/3	0/3
Propionibacterium acnes	0/4	0/4	0/4	0/4
EBV	0/5	0/5	0/5	0/5
CMV	0/10	0/10	0/10	0/10
HSV types 1 and 2	0/8	0/8	0/8	0/8
Parvovirus B19	0/4	0/4	0/4	0/4

[a] DNA probes hybridizing with *C. albicans*, *C. tropicalis*, *C. parapsilosis*, *T. glabrata*, and *C. krusei.*

[b] This DNA probe hybridizes with *A. fumigatus*, *A. flavus*, and *A. versicolor.*

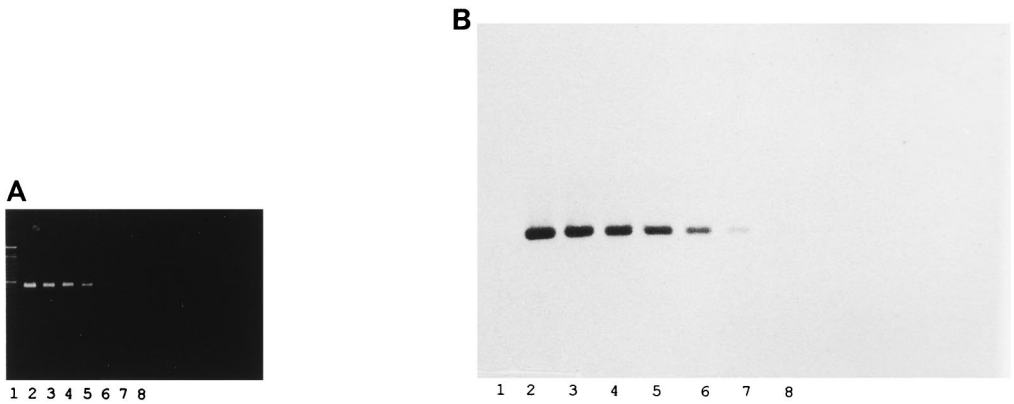

FIG. 1. Sensitivity testing. Different amounts of genomic *C. albicans* DNA were amplified with the described primer pair, and amplicons were detected by gel electrophoresis and ethidium bromide staining (A) or by slot blot hybridization (B) with a digoxigenin-labeled internal *C. albicans*-specific DNA probe. Lanes: 1, 100-bp ladder; 2 to 6, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, and 10 fg of *C. albicans* DNA, respectively; 8, negative control.

(iii) Specificity and reproducibility. A wide range of template DNA (up to 100 ng) extracted from various clinical isolates of nonfungal pathogens and human cells remained negative by amplification with the defined primer pairs: *C. perfringens*, *B. fragilis*, *B. cereus*, *P. aeruginosa*, *N. asteroides*, *L. monocytogenes*, *S. aureus*, *S. epidermidis*, *S. mitis*, *S. sanguis*, *K. pneumoniae*, *E. cloacae*, *E. coli*, *B. burgdorferi*, *P. acnes*, EBV, CMV, HSV types 1 and 2, HHV6, parvovirus B19, human fibroblasts, and lung and liver biopsy specimens (Table 2). Hybridization of the DNA samples extracted from several clinical isolates and laboratory strains of these nonfungal pathogens or human cells with all five *Candida* DNA probes and the two *Aspergillus* DNA probes also revealed negative results (Table 2).

(iv) Species- and genus-specific hybridization. Species-specific hybridization with the designed oligonucleotide probes was demonstrated for *C. albicans*, *C. tropicalis*, and *C. parapsilosis*. Despite sequence homologies of up to 90% of the genus- and species-specific oligonucleotide probes to other fungal species, no cross-reaction was observed under stringent hybridization conditions and washing procedures. Because of a sequence homology of 100%, the probe for *T. glabrata* cross-reacted with *C. tropicalis* and the probe for *C. krusei* cross-reacted with *C. parapsilosis*. By sequential hybridization with the DNA probes for *C. albicans*, *C. tropicalis*, *T. glabrata*, *C. parapsilosis*, and *C. krusei*, however, all of these fungal pathogens could be differentiated and identified (Fig. 3).

The *Aspergillus* probe, which was used for genus-level identification, hybridized with all *Aspergillus* spp. tested. Because the *Aspergillus* probe showed 100% identity with *H. capsulatum*, hybridization with this probe was additionally tested with the probe specifically hybridizing with *A. fumigatus*, *A. flavus*, and *A. versicolor*, with positive results (Tables 1 and 2).

Apart from the cross-hybridization described above and in Tables 1 and 2, no hybridization with the *Candida* and *Aspergillus* oligonucleotides was observed with DNA amplified from other fungal species (Table 2).

Screening of blood samples. All blood samples obtained from patients and controls were assayed for the presence of a band on the ethidium bromide-stained gel as well as for hybridization with the DNA probes specific for *C. albicans* and *Aspergillus* spp. (Fig. 2). Hybridization with the DNA probes for non*C.-albicans Candida* spp. was performed only when a band was present, and no hybridization with the DNA probes for *C. albicans* and *Aspergillus* spp. was documented. By using the DNA extraction method described above, all of the DNA samples extracted from the blood specimens tested in this study were amplifiable, as demonstrated by successful amplification of a DNA sequence of the human HLA class I gene (8). Hybridization was shown for all samples that were positive in the gel. For all except two hybridization-positive samples, a band was documented in the ethidium bromide-stained gel.

All group A ($n = 165$) and group B ($n = 102$) blood samples were PCR negative. Only 3 of 189 blood samples from three patients in group C were PCR positive: two for *C. albicans* and one for an *Aspergillus* sp. No further blood samples from these patients tested positive by PCR, nor were any of the blood samples positive by culture. None of these three patients developed IFI, and all improved without receiving antifungal therapy. All 112 blood samples obtained from patients in groups B and C with documented bacteremia and/or CMV viremia were PCR negative.

Patients with documented invasive fungal disease. Two blood samples were drawn prior to the initiation of antifungal therapy from all but one patient, in whom IFI was later documented. All patients with documented IFI were found to be PCR positive (Tables 3 and 4) prior to the initiation of antifungal therapy (Fig. 4 and 5). The single blood sample obtained from one patient with IFI prior to antifungal therapy was PCR positive.

The sensitivity of the PCR assay based on a single blood

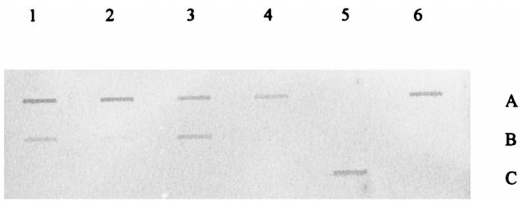

FIG. 2. Testing of clinical specimens. Slot blot hybridization with the *Aspergillus* genus-specific DNA probe of amplicons obtained from the following: A1 to A4, dilution steps of genomic *Aspergillus* DNA (100 pg, 10 pg, 1 pg, and 100 fg respectively) A5, A6, B1 to B6, and C1 to C4, sequential blood samples obtained from a patient with invasive aspergillosis during the first (A5 and A6), second (B1 and B2), and third (B3 and B4) weeks of treatment and following (B5, B6, and C1 to C4) successful antifungal treatment; C5, positive control (10 pg of *Aspergillus* DNA); C6, negative control (reagents control). Hybridization in the slot blot hybridization assay was performed with a digoxigenin-labeled internal oligonucleotide specific for *A. fumigatus*, *A. flavus*, and *A. versicolor.*

TABLE 3. Results for patients with invasive candidiasis or candidemia[a]

Patient no.	Clinical feature	PCR assay	Culture from:		No. of positive specimens/total no. of blood specimens by PCR screening at the following times prior to or during therapy:				Outcome
			Blood sample	Tissue biopsy specimen	Prior to	1st wk	2nd wk	3rd wk	
1	FUO	*C. albicans*	*C. albicans*	ND	2/2	1/3	0/1	ND	Alive
2	FUO	*C. albicans*	*C. albicans*	*C. albicans*[b]	2/2	1/1	1/1	ND	Died
3	FUO	*C. tropicalis*	*C. tropicalis*	ND	2/2	1/2	1/3	0/3	Alive
4	Pneumonia	*C. albicans*	Neg.	*C. albicans*[b]	1/1	1/1	1/1	ND	Died
5	Hepatospl. Cand.	*C. albicans*	Neg.	*C. albicans*[c]	1/2	2/3	0/2	ND	Alive
6	Lung abscess	*C. albicans*	Neg.	*C. albicans*[d]	2/2	1/3	1/2	0/3	Alive
7	Hepatospl. Cand.	*C. albicans*	Neg.	*C. albicans*[c]	2/2	1/2	ND	ND	Alive
8	FUO	*C. albicans*	*C. albicans*	ND	1/2	3/3	1/2	2/2	Died

[a] Abbreviations: FUO, fever of unknown origin; hepatospl. Cand., hepatosplenic candidiasis; ND, not done; Neg., negative.
[b] Postmortem analysis, disseminated invasive candidasis.
[c] Liver biopsy.
[d] Lung biopsy.

sample drawn prior to antifungal therapy was 88%, but it increased to 100% when two blood samples were analyzed.

We had planned to draw three blood samples weekly during antifungal therapy (intravenous amphotericin B, $n = 18$; fluconazole, $n = 3$). This could be achieved, however, only for 13 of the 21 patients due to early death (2 during the first week of therapy and 5 during the second week of therapy) or logistic difficulties. Following initiation of antifungal therapy, the percentage of PCR-positive samples decreased (Fig. 4 and 5). Among the 10 patients surviving IFI, PCR assay results became persistently negative after 14 days of antifungal therapy (Fig. 5), but they remained positive for the majority of blood samples taken during antifungal therapy from the 11 patients who died of IFI (Fig. 5).

All culture-positive blood samples from four patients (three infected with *C. albicans* and one infected with *C. tropicalis*) were also PCR positive. PCR identified the fungal pathogen that was eventually documented by histopathology and culture as causing IFI in all patients (Tables 3 and 4). The same pathogen was identified in all serial samples from each patient except two patients with invasive pulmonary aspergillosis; for these two patients, two consecutive blood samples were simultaneously positive for *C. albicans* and *Aspergillus* DNAs.

Time sequence of clinical manifestations, culture, and PCR results. For 8 of 13 patients with invasive pulmonary aspergillosis, PCR detected *Aspergillus* DNA a median of 4 days (range, 4 to 8 days) prior to documentation of pulmonary infiltrates, and for 5 patients PCR detected *Aspergillus* DNA simultaneously with the documentation of pulmonary infiltrates. All of the blood samples obtained from patients with invasive aspergillosis, including all PCR-positive samples, were culture negative. For four patients with radiologically documented manifestations of invasive candidiasis (two with lung infiltrates and two with hepatosplenic candidiasis), a positive PCR result preceded radiological findings by a median of 4 days (range, 4 to 7 days). For four patients with culture-proven candidemia, a positive PCR result remained positive for up to 14 days (median, 8 days) after blood cultures had become negative.

DISCUSSION

The largest criticism directed against the use of PCR to detect fungal DNA is the concern that, since molds are so prevalent in the environment and are such common contaminants, they may invalidate the use of PCR because of the risk of contamination. The data presented here describe the am-

TABLE 4. Results for patients with invasive aspergillosis[a]

Patient no.	Clinical feature(s)	Culture from:		No. of positive specimens/total no. of blood specimens by PCR screening at the following times prior to or during therapy:				Outcome
		BAL sample	Tissue biopsy	Prior to	1st wk	2nd wk	3rd wk	
9	Pulmon. infilt. + brain abscess	ND	*A. fumigatus*[b]	2/2	2/3	1/2	ND	Died
10	Pulmon. infilt.	*A. fumigatus*	ND	1/2	1/3	1/2	0/1	Alive
11	Pulmon. infilt. + brain abscess	*A. fumigatus*	*A. fumigatus*[b]	2/2	1/1	ND	ND	Died
12	Pulmon. infilt.	*A. fumigatus*	*A. fumigatus*[b]	1/2	2/3	1/1	ND	Died
13	Pulmon. infilt. + brain abscess	ND	*A. fumigatus*[b]	2/2	2/3	1/2	ND	Died
14	Pulmon. infilt. + brain abscess	*A. fumigatus*	*A. fumigatus*[b]	2/2	1/2	1/2	ND	Died
15	Pulmon. infilt.	*A. fumigatus*	ND	2/2	1/2	0/2	ND	Alive
16	Pulmon. infilt.	*A. fumigatus*	*A. fumigatus*[b]	2/2	2/3	1/1	ND	Died
17	Pulmon. infilt.	*A. fumigatus*	ND	2/2	2/3	0/2	0/2	Alive
18	Pulmon. infilt.	*A. fumigatus*/*A. flavus*	ND	1/2	2/3	1/3	0/1	Alive
19	Pulmon. infilt.	*A. fumigatus*	*A. fumigatus*[b]	2/2	2/3	0/1	1/1	Died
20	Pulmon. infilt.	*A. fumigatus*	ND	2/2	2/3	0/3	0/2	Alive
21	Pulmon. infilt.	*A. fumigatus*/*A. flavus*	*A. fumigatus*[b]	2/2	2/3	1/1	1/2	Died

[a] All blood samples were culture negative. Abbreviations: Pulmon. infilt., pulmonary infiltrate; ND, not done; BAL, bronchoalveolar lavage.
[b] Postmortem analysis, analysis of lung biopsy specimen with or without analysis of brain biopsy specimen.

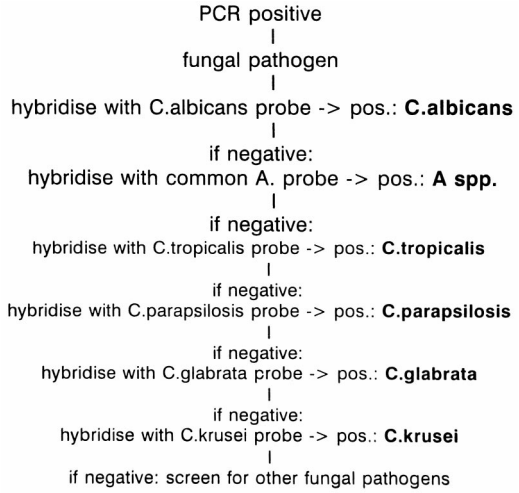

FIG. 3. PCR-based detection and differentiation of various fungal pathogens. By following an algorithm based on the frequency of the suspected isolates, the species-specific DNA probes were used sequentially for species differentiation. A., *Aspergillus* spp.

plification of DNA extracted from 85 bacterial and viral isolates, from various human cells and tissues, and from 134 fungal isolates demonstrating fungus-specific amplification.

An assay with high sensitivity is essential for the detection of fungal pathogens in blood, particularly in patients with hepatosplenic candidiasis and invasive aspergillosis, whose blood samples are rarely culture positive (7, 33). The lower limit of detection of this PCR assay in which a multicopy gene is amplified is 1 CFU/ml of blood. To achieve this high sensitivity of detection, especially for *Aspergillus* spp., the DNA extraction method was optimized by heating-alkaline denaturation-lysis, which markedly increased the yield of DNA, particularly from *Aspergillus* species, and by amplifying a gene found in multiple (>100) copy numbers. Furthermore, hybridization of the amplicons with a labeled internal oligonucleotide plus alkaline denaturation for improved hybridization further increased the sensitivity of the assay. A comparable sensitivity for the detection of isolates has also been reported for nested PCR assays (10); however, application of the sensitive nested PCR assay to clinical samples has not been described. PCR assays amplifying

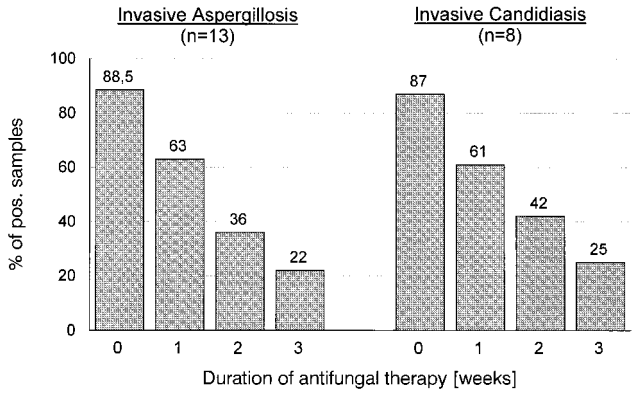

FIG. 4. Results of PCR screening of patients with IFI. The numbers of PCR-positive and PCR-negative blood samples obtained prior to (0 weeks) and during the first (1 week), second (2 weeks), and third (3 weeks) weeks of antifungal therapy are presented for 13 patients with invasive aspergillosis and 8 patients with invasive candidasis.

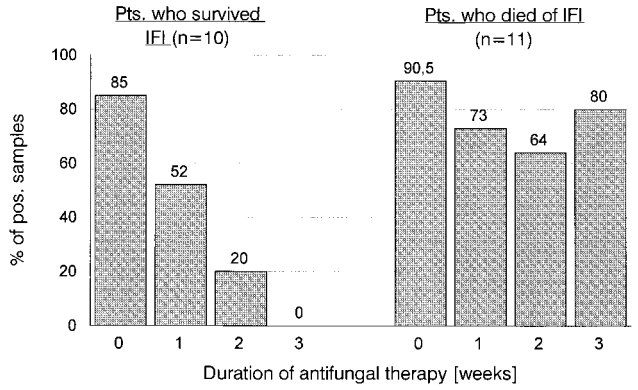

FIG. 5. PCR monitoring of patients receiving antifungal therapy for IFI. The numbers of PCR-positive (shaded bars) and PCR-negative (open bars) blood samples obtained from 10 patients responding and 11 patients not responding to antifungal therapy for IFI are presented. The samples were obtained either prior to (0 weeks) or during the first (1 week), second (2 weeks) and third (3 weeks) weeks of antifungal therapy.

target sequences of the 18S rRNA gene have been described before (6, 19, 20, 24, 26), and all showed a similarly high sensitivity in vitro compared to the sensitivity of the assay described here. When applied to the screening of experimentally infected animals, some of these assays (6, 19, 20) have shown a higher sensitivity than culture assays for the detection of fungal pathogens. In one report, testing of blood samples spiked with *C. albicans* also revealed a very high sensitivity (26). Specificity was reported to be high, and detection of more than one fungal pathogen could be achieved (19, 20, 24, 26). However, only very limited application of PCR assays based on the amplification of a sequence of the 18S rRNA gene to clinical materials has been reported up to now.

The DNA extraction procedure described here allows for a reduction of the amount of human DNA, thus enriching for fungal DNA in the final DNA sample. Therefore, the very high sensitivity of this assay can also be provided when screening blood samples.

Identification of the fungal species is essential for appropriate clinical decision making concerning both the significance of a particular isolate and the dosage and duration of antifungal therapy. Culture and microbiological determination of the species of fungi from clinical material usually require up to several days. By using the oligonucleotide probe hybridization assay described here, DNA extraction and amplification of the PCR products and determination of the fungal species can be performed within 12 h. A more rapid DNA extraction procedure and simultaneous hybridization with all the DNA probes applying new hybridization techniques will afford even faster detection and differentiation of fungal species. Alternatively, PCR-single-strand conformational polymorphism analysis has been successfully applied to the differentiation of *Aspergillus* species (35). Unfortunately, differentiation of *Candida* species could not be achieved with the gene region amplified, and application to clinical specimens has not been demonstrated.

A large number of blood samples ($n = 601$) obtained from a control group and three clearly defined patient groups were analyzed simultaneously by PCR and culture assay in order to assess the clinical applicability of the assay. PCR demonstrated a high specificity for screening blood samples with all 165 blood samples from healthy controls, and 288 of 291 samples from patients with febrile neutropenia, but without IFI, tested PCR negative. Only 3 of 189 blood samples obtained from 3 of the 36 patients colonized with *Candida* species were PCR positive.

Similarly, there was a low rate of positivity in the *Candida* enolase test (34), with 6% of colonized patients being positive, and in an animal model (6), in which even all blood samples from mice colonized with *Candida* species were PCR negative.

Testing of blood samples allowed for the detection and identification of the fungal pathogen later documented by histopathology and culture as the cause of invasive infection for all patients analyzed. Only in two patients with documented colonization with *Candida* species but later histopathologically proven invasive aspergillosis were *Candida albicans* and *Aspergillus* DNAs simultaneously detected in two consecutive blood samples. This indicates the potential of the assay to detect infections caused by more than one fungal pathogen and to identify the fungal species present. The reliable identification of the causative pathogen and the consistency of the species in serial samples identified by the assay additionally confirm the high specificity of the assay in screening clinical material.

A sensitivity of 100% was attained by testing multiple specimens from all patients with invasive candidiasis ($n = 8$) and aspergillosis ($n = 13$). The sensitivity of a single PCR assay performed with blood samples obtained prior to the initiation of antifungal therapy was 88%. Multiple tests improved the sensitivity of detection for other diagnostic assays as well, such as the *Candida* enolase test (34) and several *Aspergillus* antigen tests (25, 28).

During antifungal therapy the percentage of PCR-positive blood samples decreased. Thus, for the sensitive detection of fungal pathogens, blood samples should be screened prior to the initiation of antifungal therapy.

Because only samples from patients with documented IFI and patients without clinical evidence of IFI were included in this analysis, in an ongoing study, patients at risk of developing IFI are prospectively screened by PCR during neutropenia to define the negative and positive predictive values of this assay for the development of IFI in high-risk patients.

At least as shown for 10 of the 21 patients with IFI, the disappearance of fungal DNA from the blood correlated with successful therapy, indicating the clearance of fungal DNA from the blood during or following antifungal treatment. In contrast, 11 patients who finally died of IFI remained PCR positive during antifungal therapy, indicating the potential of monitoring the in vivo efficacy of antifungal therapy by PCR. However, due to the low numbers of blood samples analyzed during the second and third weeks of therapy in both responding and nonresponding patients, application of the assay to a larger number of patients receiving antifungal therapy is essential to further assessing the value of the technique in monitoring antifungal therapy.

In conclusion, the PCR assay designed and tested as described here provides a high sensitivity and specificity for the detection of fungal DNA in blood samples and rapidly identifies most *Candida* and *Aspergillus* species. Thus, the vast majority of clinically relevant fungal pathogens in the immunocompromised patient are detected by this assay.

The screening program which tested the assay with a limited number of serial specimens was successful, indicating that this assay might have some value in screening high-risk patients and in monitoring antifungal therapy.

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REFERENCES

1. **Banerjee, S. N., T. E. Emori, D. H. Culver, et al.** 1991. Secular trends in nosocomial primary bloodstream infections in the United States, 1980–1990. National Nosocomial Infections Surveillance System. Am. J. Med. **91**(Suppl. 3B)**:**86S–89S.
2. **Bodey, G. P.** 1988. The emergence of fungi as major hospital pathogens. J. Hosp. Infect. **11**(Suppl. A)**:**411–426.
3. **Bretagne, S., J. M. Costa, A. Marmorat-Khuong, F. Poron, C. Cordonnier, M. Vidaud, and J. Fleury-Feith.** 1995. Detection of *Aspergillus* species DNA in bronchoalveolar lavage samples by competitive PCR. J. Clin. Microbiol. **33:**1164–1168.
4. **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.
5. **Buckley, H. R., M. D. Richardson, E. G. V. Evans, and L. J. Wheat.** 1992. Immunodiagnosis of invasive fungal infection. J. Med. Vet. Mycol. **30**(Suppl. 1)**:**249–260.
6. **Deventer, A. J. M., W. H. F. Goessens, A. van Belkum, H. J. A. van Vliet, E. W. M. van Etten, and H. A. Verbrugh.** 1995. Improved detection of *Candida albicans* by PCR in blood of neutropenic mice with systemic candidiasis. J. Clin. Microbiol. **33:**625–628.
7. **Duthie, R., and D. W. Denning.** 1995. Aspergillus fungemia: report of two cases and review. Clin. Infect. Dis. **20:**598–605.
8. **Einsele, H., G. Ehninger, M. Steidle, A. Vallbracht, M. Müller, H. Schmidt, J. G. Saal, H. D. Waller, and C. A. Müller.** 1991. Polymerase chain reaction to evaluate antiviral therapy for cytomegalovirus disease. Lancet **ii:**1170–1172.
9. **Fraser, V. J., M. Jones, J. Dunkel, et al.** 1992. Candidemia in a tertiary care hospital: epidemiology, risk factors, and predictors of mortality. Clin. Infect. Dis. **15:**414–421.
10. **Haynes, K. A., T. J. Westerneng, J. W. Fell, and W. Moens.** 1995. Rapid detection and identification of pathogenic fungi by polymerase chain reaction amplification of large subunit ribosomal DNA. J. Med. Vet. Mycol. **33:**319–325.
11. **Holmes, A. R., R. D. Cannon, M. G. Shepherd, and H. F. Jenkinson.** 1994. Detection of *Candida albicans* and other yeast in blood by PCR. J. Clin. Microbiol. **32:**228–231.
12. **Hopfer, R. L., P. Walden, S. Setterquist, and W. E. Highsmith.** 1993. Detection and differentiation of fungi in clinical specimens using the polymerase chain reaction (PCR) amplification and restriction enzyme analysis. J. Med. Vet. Mycol. **31:**65–74.
13. **Horn, R., B. Wong, T. Kiehn, and D. Armstrong.** 1985. Fungemia in a cancer hospital: changing frequency, earlier onset and results of therapy. Rev. Infect. Dis. **7:**646–655.
14. **Jones, S., G. White, and P. R. Hunter.** 1994. Increased phenotype switching in strains of *Candida albicans* associated with invasive infections. J. Clin. Microbiol. **32:**2869–2870.
15. **Jordan, J. A.** 1994. PCR identification of four medically important Candida species by using a single primer pair. J. Clin. Microbiol. **32:**2962–2967.
16. **Kan, V. L.** 1993. Polymerase chain reaction for the diagnosis of candidemia. J. Infect. Dis. **168:**779–783.
17. **Komshian, S. V., A. K. Uwaydah, J. D. Sobel, and L. R. Crane.** 1989. Fungemia caused by Candida species and Torulopsis glabrata in the hospitalized patient: frequency, characteristics, and evaluation of factors influencing outcome. Rev. Infect. Dis. **11:**379–390.
18. **Kwok, S., and R. Higuchi.** 1989. Avoiding false-positives with PCR. Nature **339:**237–238.
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. **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.
21. **Meyers, J. D.** 1990. Fungal infections in bone marrow transplant patients. Semin. Oncol. **17**(Suppl. 6)**:**10–13.
22. **Miyakawa, Y., T. Mabuchi, K. Kagaya, and Y. Fukazawa.** 1992. Isolation and characterization of a species-specific DNA fragment for detection of *Candida albicans* by polymerase chain reaction. J. Clin. Microbiol. **30:**894–900.
23. **Morrison, A. J., C. V. Freer, M. A. Searcy, S. M. Landry, and R. P. Wenzel.** 1986. Nosocomial blood stream infections. Secular trends in a statewide surveillance program in Virginia. Infect. Control **7:**550–553.
24. **Niesters, H. G. M., W. H. F. Goessens, J. F. M. G. Meis, and W. G. Y. Quint.** 1993. Rapid polymerase chain reaction-based identification assays for *Candida* species. J. Clin. Microbiol. **31:**904–910.
25. **Patterson, T. F., P. Minitier, J. E. Patterson, J. M. Rappeport, and V. T. Andriole.** 1995. Aspergillus antigen detection in the diagnosis of invasive aspergillosis. J. Infect. Dis. **171:**1553–1558.
26. **Polanco, A. M., J. Rodriguez-Tudela, and J. V. Martinez-Suárez.** 1995. Detection of pathogenic fungi in human blood by the polymerase chain reaction. Eur. J. Clin. Microbiol. Infect. Dis. **14:**618–621.
27. **Reddy, L. V., A. Kumar, and V. P. Kurup.** 1993. Specific amplification of Aspergillus DNA by polymerase chain reaction. Mol. Cell. Probes **7:**121–126.
28. **Rogers, T. R., K. A. Haynes, and R. A. Barnes.** 1990. Value of antigen

detection in predicting invasive pulmonary aspergillosis. Lancet **336:**1210–1213.
29. **Schaberg, D. R., D. H. Culver, and R. P. Gaynes.** 1991. Major trends in the microbial etiology of nosocomial infection. Am. J. Med. **91**(Suppl. 3B)**:**72S–75S.
30. **Schroppel, K., M. Rotman, R. Galask, K. Mac, and D. R. Soll.** 1994. Evolution and replacement of Candida albicans strains during recurrent vaginitis demonstrated by DNA fingerprinting. J. Clin. Microbiol. **32:**2646–2654.
31. **Spreadbury, C., D. Holden, A. Aufauvre-Brown, B. Bainbridge, and J. Cohen.** 1993. Detection of *Aspergillus fumigatus* by polymerase chain reaction. J. Clin. Microbiol. **31:**615–629.
32. **Tang, C. M., D. W. Holden, A. Aufauvre-Brown, and J. Cohen.** 1993. The detection of Aspergillus spp. by the polymerase chain reaction and its evaluation in bronchoalveolar lavage fluid. Am. J. Respir. Dis. **148:**1313–1317.
33. **Thaler, M., B. Pastakia, T. H. Shawker, T. O'Leary, and P. A. Pizzo.** 1988. Hepatic candidiasis in cancer patients: the evolving picture of the syndrome. Ann. Intern. Med. **108:**88–100.
34. **Walsh, T. J., J. W. Hathorn, J. D. Sobel, et al.** 1991. Detection of circulating Candida enolase by immunoassay in patients with cancer and invasive candidiasis. N. Engl. J. Med. **324:**1026–1031.
35. **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.
36. **Whimbey, E., T. E. Kiehn, P. Brannon, A. Blevins, and D. Armstrong.** 1987. Bacteriemia and fungemia in a patient with neoplastic diseases. Am. J. Med. **82:**723–730.