The incidence of opportunistic fungal infections has increased dramatically in the past few decades, especially in immunocompromised hosts (13). Candidiasis and aspergillosis frequently occur in patients with hematological malignancies, who experience periods of prolonged granulocytopenia caused by intensive cytostatic chemotherapy (3, 13, 27). In these patients, colonization of mucosal surfaces by endogenous Candida spp. is often followed by invasion of the vascular space, which carries a high risk of disseminated candidiasis. For this reason, most neutropenic patients with fever that fails to respond to single or multiple broad-spectrum antibiotic therapy are empirically treated with amphotericin B (10, 12, 31).

Fungemia is, in fact, one of the most feared complications in these patients, and their survival is strictly dependent on early antifungal treatment and prompt resolution of their neutropenia, which is considered the most important risk factor (3, 4, 9, 10, 13, 17–19, 27). In the majority of patients, the fungemia is caused by Candida albicans, although in the last 10 years there has been an increase in the incidence of infection with a non-C. albicans Candida etiology (26). Early diagnosis of fungemia is difficult to obtain by conventional procedures like blood culture or those that depend upon a functioning immune system (4, 7, 14, 15, 18, 25, 26, 30), and far too often, deep fungal infections are diagnosed only at autopsy (1, 8, 16, 20). Although considerable progress in the detection of fungemia has recently been made, conventional fungal blood cultures still require 1 to 2 weeks of incubation, and no one system can be considered sensitive enough to detect all candidiasis (4, 7, 14, 15, 25, 30). Early detection of Candida species in blood could improve the survival of patients with hematological malignancies by allowing the initiation of specific antifungal treatment while the fungal biomass is still low (10, 12, 27, 31). A more rapid and sensitive method would also be useful for the monitoring of treatment of these infections, which is based in many cases on the administration of amphotericin B, which has many adverse side effects.

Recently, we developed a PCR method to amplify a 350-bp segment of the P-450 lanosterol 14α-demethylase gene, and with appropriate restriction enzyme analysis (REA) of the resultant amplicons, this approach can be used to identify the Candida spp. most commonly involved in human infections. In preliminary studies, PCR-REA was capable of detecting as little as 200 fg of Candida DNA (21). In the present study we compared our method with a method that uses a conventional automated blood culture system for the early detection of candidemia. The patients whom we examined had hematological malignancies (in most cases acute myeloid leukemia), significant neutropenia, or fever of unknown origin (FUO) that was unresponsive to 3 days of therapy with broad-spectrum antibiotics.

**MATERIALS AND METHODS**

**Patients.** The study population was composed of 72 patients (42 males and 30 females) with hematological malignancies. The patients were consecutively evaluated between May 1996 and January 1997 for FUO that had failed to respond to broad-spectrum antibiotic therapy. Twenty-seven had acute myeloid leukemia, 5 had acute lymphoid leukemia, 18 had lymphomas, 6 had Hodgkin’s disease, 8 had multiple myeloma, 2 had chronic myeloid leukemia, and 2 had aplastic anemia; the remaining four had mycosis fungoides, hairy cell leukemia, chronic lymphoid leukemia, and malignant histiocytosis, respectively. The ages of the patients ranged from 15 to 80 years, with a median age of 49 years. All of the febrile episodes occurred while the patients were receiving chemotherapy, and over half (51.3%) were associated with the induction phase of treatment.

At the time of their enrollment in the study all but one patient (the patient with mycosis fungoides) had absolute neutrophil counts of <0.5 × 10⁹/liter. Blood sample collection began after the FUO had been unsuccessfully treated for 3 to 4 days with a cephalospiran plus an aminoglycoside with or without a glycopeptide. Each specimen (23 ml) was collected by aseptic venipuncture; 3 ml (collected in tubes containing EDTA) was used for PCR-REA studies, and the remaining 20 ml was used for blood cultures. This process was repeated daily until the fever had resolved or its cause was identified (i.e., bacteremia, pneu-
monia, or invasive fungal disease). The total number of blood samples studied was 217.

Additional surveillance cultures (pharynx, sputum, stool, and urine) were also performed on patients, and when clinical signs of deep fungal infections persisted, other specimens were submitted for histological study (e.g., skin biopsy) and/or culture (e.g., pericardial or pleural fluids, central venous catheter tip, bronchoalveolar lavages). Ten of the 72 patients died, and autopsies were performed on 7 patients.

Antifungal treatment. All of the patients had been placed on prophylactic therapy with oral amphotericin B (0.5 g every 8 h) at the beginning of chemother-apy. After the first blood specimen had been collected, this therapy was discontinued and intravenous amphotericin B therapy was started at a dosage of 0.8 mg/kg of body weight/day. In the presence of clinical and laboratory findings compatible with disseminated candidiasis or any other deep fungal disease, the dosage of intravenous amphotericin B was increased to a maximum of 1.2 mg/kg/day (17). Treatment was continued (unless death occurred) until the patient was afebrile with an absolute neutrophil count of >0.5 × 10^9/liter.

Blood cultures. A total of 8 to 10 ml of blood from each draw (23 ml) was inoculated into a Mycosis IC/F bottle (Becton Dickinson, Cockeysville, Md.), which contains a medium specifically designed to support fungal growth; another 10 ml was used for conventional aerobic and anaerobic bacterial cultures (Plus/aerobic/S* and Plus/anaerobic/F* medium, respectively; 5 ml for each bottle). All of the bottles were incubated in a BACTEC 9240 automated system. Cultures were considered negative when no growth was detected after 7 days (Plus/aerobic/S* and Plus/anaerobic/F* medium bottles) or 14 days (Mycosis IC/F* bottle) of incubation. When microbial growth was detected by the system, microscopic examinations by Gram staining were performed, and subcultures were made on Bacto Candida Growth agar, chocolate agar, MacConkey agar, and blood agar. Bacteria and yeasts were identified by conventional biochemical tests and (for yeasts) micromorphology on Dalmau agar plates (22).

Surveillance and other specimen cultures. Pharyngeal swabs, midstream urine, sputum, and stool samples were collected once a week and were cultured for fungi by standard procedures (22). When clinical signs of deep fungal infections were present, cutaneous biopsy, bronchoalveolar lavage, and/or pleural or pericardial fluid specimens were also cultured by standard procedures (22). Yeasts were identified by biochemical characteristics and micromorphology, while the slide culture technique was used to identify filamentous fungi. Histopathologic observation was performed for autopsy specimens as well as for biopsy specimens. The following criteria were used to define yeast colonization and disseminated candidiasis: (i) superficial colonization, on histopathologic examination, of pharyngeal swabs or stool; (ii) mucocutaneous colonization, three or more pharyngeal swabs were positive for the same yeast in a patient with clinical manifestations of oral candidiasis or at least three different specimens (e.g., urine, sputum, and stool) were positive for the same yeast; and (iii) disseminated candidiasis, histopathologic evidence of organ involvement, ultrason sound evidence of chronic hepatosplenic candidiasis, and/or positivity for the same yeast species in two or more cultures of blood, tissue biopsy, pleural or pericardial fluid, or bronchoalveolar lavage specimens was found.

Processing of blood samples for PCR. Three milliliters of blood from each draw was collected in tubes containing EDTA. This sample was immediately taken to the laboratory, where it was diluted with 3 ml of sterile phosphate-buffered saline (PBS) (pH 7.2), and this was added to 3 ml of PBS containing 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, Aurora, Ohio), and centrifuged at 1,200 × g for 30 min to eliminate erythrocytes and to collect leukocytes and yeast cells. After two washes in PBS (30 ml), the cells (leukocytes and yeasts) were suspended in 20 ml of 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, Aurora, Ohio), and the mixture was incubated for 2 h at 37°C. The spheroplasts were then lysed with proteinase K (0.5 mg/ml) and sodium dodecyl sulfate (0.2%). After two extractions with phenol-chloroform-isomyl alcohol (25:24:1), the DNA was purified on a Sephadex G50 column and precipitated with cold ethanol. The synthesis of oligonucleotides used as primers P450, 5'-ATG ACT CAT CAA GAA ACT TCT AA-3' and primer P450, 5'-TAA CCT GGA GAA ACG AAA AC-3' (22). The PCR was performed in a Thermal Cycler 2400 (Perkin-Elmer, Foster City, Calif.). The first cycle was 4 min of denaturation at 94°C, 1 min of annealing at 54°C, and 1 min and 30 s of extension at 72°C. This was followed by 40 cycles, each consisting of 45 s of denaturation at 94°C, 1 min of annealing at 54°C, and 1 min and 30 s of extension at 72°C. For each PCR run negative and positive controls were also included. Several negative controls consisting of water were included in each run; total DNA from C. albicans (2 pg of DNA) was used as a positive control. Each step of our protocol was performed in separate rooms to prevent sample contamination. 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 NaCl, 1 mM EDTA [pH 8.4]), the gel was stained with ethidium bromide, and the bands were visualized with UV light (302 nm). All amplicons were subjected to REA with Sau3AI, HincII, and NarI (Boehringer Mannheim) according to the manufacturer’s instructions. After digestion, the DNA was electrophoresed in an agarose gel (4% [3% SeaKem GTG and 1% NuSieve] in TAE buffer) at 70 V for 2 h at room temperature, stained with ethidium bromide (0.5 μg/ml), and photographed under UV light. The REA patterns thus obtained identified the amplified fungal DNA specimens as C. albicans, C. tropicalis, C. glabrata, C. krusei, C. kefyr, C. parapsilosis, or C. guilliermondii. As demonstrated previously (21), the PCR-REA method was able to detect 5 CFU of Candida DNA per ml of blood.

Statistical analysis. Diagnoses regarding the fungal and/or bacterial etiology of the febrile episodes were based entirely upon clinical data, culture findings, histology findings, and for three patients, autopsy findings. Patients whose symptoms and test results met the criteria for disseminated candidiasis described above were considered true positives, and the ability of the PCR-REA method to predict or exclude this outcome was calculated with Epi-Info software (version 6.04, January 1997, Centers for Disease Control and Prevention, Atlanta, Ga., and World Health Organization, Geneva, Switzerland).

RESULTS

PCR-REA findings versus fungal blood cultures. Candidal DNA was detected by PCR in one or more blood samples from 31 patients. The number of blood specimens tested per patient in the PCR-positive subgroup ranged from 1 to 11 (mean, 4.6 specimens). The number of specimens tested for the 41 patients who were PCR negative was much lower (mean, 1.8) because the majority (34 of 41) became afebrile 1 or 2 days after enrollment in the study. For all 31 PCR-positive patients, Candida DNA was detected in the first blood specimen collected, and for 11 patients, the species was identified in two to seven subsequently drawn samples. For 20 patients there was only one PCR-positive blood sample; for 8 of these patients, this sample was the only one studied (due to patient death or early resolution of fever), but for 12 of these 20 patients one or more subsequent specimens were PCR negative. Species were identified by REA of the amplicons and were as follows: C. albicans (24 patients), C. glabrata (2 patients), C. tropicalis (2 patients), and C. kefyr (3 patients).

Only four patients were positive for yeasts by blood culture. All of the blood samples that yielded positive results by PCR were also positive by PCR, and in all four patients, the species identified in blood cultures was the same as that revealed by PCR-REA (C. albicans in three patients and C. tropicalis in one patient). In two patients, however, the first specimen drawn (which was positive for yeasts by PCR-REA) grew only Streptococcus salivarius and Staphylococcus aureus, respectively, while cultures of blood drawn on the second and third days were both positive for C. albicans. In all four patients, detectable yeast growth in the Mycosis IC/F* bottle occurred approximately 12 h earlier than that in Plus/aerobic/S* and Plus/anaerobic/F* medium bottles did.

For the positive cultures, fungal growth was first detected after approximately 72 h of incubation, and species identification by conventional methods required another 72 h. In contrast, the PCR-REA method allowed detection and identification of yeasts from blood within 24 to 36 h.

Final diagnoses and antifungal treatment. The results of the PCR-REA were analyzed in light of the final diagnoses presented in Table 1, which were based on clinical data and culture, histology, and autopsy findings. The symptoms and test results for the 14 of the 72 patients examined ultimately met our diagnostic criteria for disseminated candidiasis (including those for 2 patients who also had evidence of bacteremia): for
8 patients on the basis of histologic examination of biopsy or autopsy material (supported by blood culture positivity in 2 patients and by ultrasound evidence of hepatosplenic involvement in 4 patients), for 5 patients on the basis of ultrasound evidence of hepatosplenic involvement (supported by blood culture positivity for 2 patients, by pleural fluid cultures for 1 patient, and by PCR-REA positivity for more than six samples from 1 patient), and for 1 patient on the basis of cultures of pericardial fluid.

Thirteen of these 14 patients were also PCR positive, including the 2 patients who were also bacteremic. For eight patients the same *Candida* species was identified in three or more samples. Only 1 of the 41 PCR-negative patients developed disseminated disease. In that patient only one blood specimen was studied because by day 2 the patient was afebrile with a normal neutrophil count. The diagnosis of disseminated candidiasis was made 35 days later on the basis of ultrasound findings of chronic hepatosplenic candidiasis.

Of the remaining 18 PCR-positive patients, 4 had pulmonary aspergillosis, 3 had bacteremia, and 1 had tuberculosis; for the remaining 10 patients, no systemic infection could be identified on the basis of clinical data or culture, histology, and/or autopsy findings (although 2 patients had severe mucocutaneous candidiasis). The majority of the PCR-positive patients showed signs of yeast colonization (superficial colonization in 7 patients and mucocutaneous colonization in 11 patients), and in all patients the colonizing species identified by traditional methods was identical to the one revealed in the blood by PCR-REA. Twenty-three of the 41 patients with negative PCR findings were colonized with yeasts, and in most patients the colonization was superficial.

In keeping with the therapeutic protocol used in our medical center for neutropenic patients, intravenous amphotericin B therapy was continued until the patient was afebrile with a neutrophil count of \( \geq 5 \times 10^9/\text{liter} \) even when blood cultures were negative for fungi. Twenty-seven of the 72 patients were taken off intravenous amphotericin B therapy after a maximum of 3 days (total dose, \( \leq 180 \) mg) because their fever had resolved and their neutrophil counts had normalized; all 27 patients were PCR negative. For 26 patients the validity of this decision was ultimately confirmed by the clinical course and laboratory data. Retrospective analysis reveals that the remaining patient, for whom the final diagnosis was disseminated candidiasis solely on the basis of sonographic evidence of hepatosplenic candidiasis, would have benefited from continued antifungal treatment.

All of the other patients (31 patients who were PCR positive and 14 patients who PCR negative) were treated intravenously with amphotericin B until the fever and neutropenia had subsided or, for 10 patients, death occurred (duration of treatment, 2 to 20 days; total median dose, 930 mg). This group included 13 of the 14 patients with disseminated candidiasis (all of whom were PCR positive), the 7 patients with aspergillosis, and the single patient with zygomycosis (in the last eight patients there was histopathologic evidence of involvement of the etiologic agents isolated from cultures). Nine other patients presented with evidence of yeast colonization (mucocutaneous colonization in three patients and superficial colonization in six patients) but no sign of visceral involvement. In the remaining 14 patients, there was no sign (apart from persistent fever) of fungal infection or colonization on the basis of the culture results or clinical findings, but for 10 of these patients, one or more blood samples was positive for *Candida* DNA by the PCR-REA. The daily dose of amphotericin B was increased to 1.2 mg/kg in 20 patients (16 PCR-positive patients and 4 PCR-negative patients) because blood cultures were positive for fungi or other signs of deep fungal disease were present.

Among the 10 patients who died, the deaths of 4 patients (2 PCR-positives patients and 2 PCR-negative patients) were attributed exclusively to the underlying malignancy. Four other deaths were attributed at least in part to disseminated candidiasis, one was attributed to aspergillosis, and one was attributed to zygomycosis.

**Statistical analysis.** The data presented above were evaluated with EpiInfo/6 software (version 6.04, January 1997) by considering as true positives the 14 patients with proven disseminated candidiasis. The sensitivity of the molecular method was 92.8% (13 of 14), whereas that of conventional fungal blood culture was 21.4% (3 of 14). The negative predictive value was 97.5% for the molecular method, whereas it was 83.8% for culture.

**DISCUSSION**

In patients with profound, persistent neutropenia like those in our study population, the presence of *Candida* species in the blood carries an extremely high risk for disseminated candidiasis, but detection of candidemia by means of fungal blood cultures is notoriously difficult, and a substantial percentage of cases of visceral candidiasis are diagnosed only at autopsy. For this reason, high-risk patients are generally subjected to prolonged, highly toxic therapy with amphotericin B, even when cultures are negative for fungi.

Other groups have reported promising results with PCR methods for the detection of fungemia (5, 6, 11, 14, 23, 28, 29, 30) in yeast-inoculated animals or in clinical specimens for which positive results were proven by culture. The present
Our findings indicate that the PCR-REA approach provides an earlier and more sensitive means of detection of candidemia than conventional blood cultures (Table 2). The molecular method yielded positive results for 13 of the 14 patients with confirmed disseminated candidiasis, which gives it a sensitivity of 92.8%. This value is not only higher than that for conventional blood cultures with fungal medium and the BACTEC automated system (21.4%) but it also exceeds that reported by Kelly et al. (15) for the isolator system (70%), which is considered the best culture system for the isolation of yeast from blood. Since the technical time needed for detection and species identification by our PCR-REA method (24 to 36 h) is also less than that required for fungal blood cultures (6 to 7 days), the molecular method can provide significantly earlier diagnosis of candidemia. For two of the four patients whose blood samples were culture positive for yeasts, the first set of blood samples grew only gram-positive cocci on culture, whereas samples from the skin. Contamination during the PCR-REA procedure is highly unlikely because of the stringent controls that we used and subsequent analyses.

Nevertheless, the question of the method’s specificity needs further investigation. According to our study design, 18 of the 72 patients whom we studied (including the 3 just mentioned) had false-positive results in that their PCR positivity did not coincide with an outcome of disseminated candidiasis. It is important to recall, however, that 10 of these 18 patients continued treatment with amphotericin B (because their fever persisted), and it is possible that at least some cases of significant candidemia (e.g., six patients for whom two or more specimens were PCR positive for the same species) were blocked by this treatment. In fact, for these patients the antifungal treatment seemed to be responsible for the resolution of fever, which occurred in the absence of neutrophil recovery. For the remaining eight patients the early resolution of the FUS or the absence of PCR positivity for subsequent specimens suggests the possibility of transient candidemia. Four of these patients had pulmonary aspergillosis, but cross-reactions by PCR must be excluded since the primers that we used did not amplify Aspergillus DNA (21). However, it is possible that naked DNA was detected by PCR due to the presence of dead and degrading yeasts within circulating phagocytes.

In contrast, the negative predictive value of the PCR-REA with respect to the development of disseminated candidiasis was quite high (97.5% versus 83.8% for blood cultures). Over half of the PCR-negative patients were taken off amphotericin B therapy after 2 to 3 days (because they were afebrile and their neutrophil counts were normal), and in spite of the lack of coverage, only one developed deep fungal disease. For this one patient with a false-negative PCR result, only one blood sample was studied because the patient’s fever resolved almost immediately after enrollment. It is possible that subsequent samples might have been positive had they been drawn. PCR negativity alone cannot justify the interruption of antifungal treatment in febrile neutropenic patients (among other things, it does not exclude non-Candida forms of fungemia), but as supporting evidence for such a decision (e.g., in a patient whose fever has subsided), it is clearly more reliable than blood culture negativity. Although it should be remembered that recovery from fungal infections is often related primarily to an improvement in the patient’s immunological status, we feel that our PCR method, due to its relative speed and sensitivity, is a potentially useful tool for the management of patients who have hematological malignancies and who are at risk for invasive candida infections.
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