Panfungal PCR Assay for Detection of Fungal Infection in Human Blood Specimens

JO-ANNE VAN BURIK, DAVID MYERSON, RANDALL W. SCHRECKHISE, and RALEIGH A. BOWDEN
Programs in Infectious Diseases and Pathology, Fred Hutchinson Cancer Research Center, Seattle, Washington

Received 14 July 1997/Returned for modification 2 October 1997/Accepted 13 January 1998

A novel panfungal PCR assay which detects the small-subunit rRNA gene sequence of the two major fungal groups was used to test whole-blood specimens obtained from a series of blood or bone marrow transplant recipients. The 580-bp PCR product was identified after amplification by panfungal primers and hybridization to a 245-bp digoxigenin-labeled probe. The lower limit of detection of the assay was approximately four organisms per milliliter of blood. Multiple whole-blood specimens from five patients without fungal infection or colonization had negative PCR results. Specimens from 11 infected patients had positive PCR results. Blood from three patients with pulmonary aspergillosis had positive PCR results; one patient's blood specimen obtained in the week prior to the diagnosis of infection by a positive bronchoalveolar lavage fluid culture result was positive by PCR, and blood specimens obtained from two patients 1 to 2 days after lung biopsy and which were sterile by culture were positive by PCR. The blood of four patients with candidemia, three patients with mixed fungal infections, and one patient with fusariosis also had positive PCR signals. The panfungal PCR assay can detect multiple fungal genera and may be used as an adjunct to conventional methods for the detection of fungal infection or for describing the natural history of fungal infection. Further studies are needed to define the sensitivity and specificity of this assay for the diagnosis of fungal infection prior to the existence of other clinical or laboratory indications of invasive fungal infection.

The prevalence of fungal infections has increased in recent years due to an increasing population of immunocompromised patients, intensive immunosuppressive chemotherapy, increasing awareness of fungal infections, and the widespread use of broad-spectrum antibiotics and central venous catheters (2). Standard methods for the diagnosis of Candida and Aspergillus fungal infections include culture and histopathology, but these methods have limited sensitivity and specificity (11, 23, 46). For example, blood cultures are positive for fewer than 50% of patients with hepatosplenic candidiasis (46) and are rarely positive for patients with invasive aspergillosis (11, 49). In addition, cultures of bronchoalveolar lavage fluid are frequently negative for patients with pulmonary aspergillosis (23), and by the time that positive cultures are obtained, disease is usually advanced.

Rapid diagnostic strategies for fungal infections include detection of antibody, antigen, or DNA. Antibody detection in bone marrow transplant (BMT) patients is limited due to unpredictable humoral responses (53). Second-generation tests for the detection of Candida and Aspergillus antigens look promising but have not been compared with culture with samples from humans (20, 34).

A PCR assay for the detection of fungal nucleic acids may be the optimal diagnostic approach because it offers the potential of (i) being more sensitive than current culture-based methods, (ii) encompassing multiple fungal genera, and (iii) being applied to a variety of specimen types. The design of fungal primers that can detect the appropriate range of fungal organisms has remained a challenge to the development of PCR assays for fungi. Most studies of the application of PCR techniques to the detection of fungal DNA have used assays with either primers (6–9, 19, 22, 24, 33, 38, 45, 48) or probes (5, 7, 12, 15, 22, 24, 31, 40) targeted to gene sequences restricted to one genus or species of fungi in order to maintain specificity. However, since the incidence of individual infecting species, e.g., Candida (10%) (32, 47), Aspergillus (5 to 15%) (21, 32, 35, 39, 41, 49), and Fusarium (<2%) (16), is relatively low and may vary from center to center, a panfungal assay is desired. This universality of nucleic acid-based methods is not possible with antigen-, antibody-, or metabolite-based assays and is practical because rRNA gene sequences are relatively conserved among euakaryotic organisms (30) and specifically among members of the fungal kingdom, including the Aspergillus and Candida species, the dimorphic fungi, the agents of zygomycosis, and Pneumocystis (4).

We used sequence analysis to develop a PCR assay reactive to the DNA of medically important fungi but unreactive to bacterial or human DNA. The panfungal primers were optimized separately for Candida albicans and Aspergillus fumigatus, the major organisms responsible for invasive yeast and mold infections, respectively. The long probe was synthesized by PCR with an internal primer pair and multiply labeled with digoxigenin. The long probe detects organisms in several subdivisions of the fungal kingdom because its long length will anneal to any product with >85% sequence homology. The known sequences are at least 85% homologous. Primary detection of the fungal PCR product must include hybridization to a probe when human blood specimens are used because human DNA can obscure the detection of a weak fungal DNA signal on agarose gels. We evaluated the assay for its ability to detect fungi in the blood of patients with and without invasive fungal disease.

MATERIALS AND METHODS

Sequence analysis for the design of the primers and probe. rRNA gene sequences from 42 organisms were accessed via the GenBank database and were
aligned by using the Wisconsin Sequence Analysis Package (Genetics Computer Group, Madison, Wis.) (10). Multiple potential primer-binding sites for the panfungal primer pair were chosen by comparing regions of Aspergillus homolo-
gous with regions of the fungal group from the fungal kingdom with the most diver-
sive sequence, Microsporum, and the two main PCR products, the human DNA sequence. The primer selection was optimized for melting temperature equivalence, lack of duplex, hairpin, or primer dimer formation, and internal stability by using OLiGO software (National Biosciences Inc., Plymouth, Minn.). Amplification conditions for A. fumigatus and C. albicans were optimized separately by a previously described method (3).

Subjects and collection of specimens. Whole-blood specimens were collected from patients at the Fred Hutchinson Cancer Research Center in Seattle, Wash., placed in Vacutainers containing 1.5 ml of acid citrate dextrose (ACD) antico-
agulant, and stored at 4°C. Written informed consent was obtained from all subjects by procedures approved by the Institutional Review Board of the Fred Hutchinson Cancer Research Center. Mouthwash specimens for fungal cultures were obtained on a weekly basis to determine colonization status. Blood cultures were performed with the BacT/Alert FAN aerobic culture bottle (Organon Teknika, Durham, N.C.) in combination with the Isolator aerobic culture tube (Wampole, Cranbury, N.J.). To obtain specimens from the days preceding in-
fec tion, patients were asked to donate 6 ml of whole-blood specimens prospect-
tively on a weekly basis. If the patient showed symptoms or signs relating to fungal disease, we attempted to collect specimens daily while the clinical workup for disease proceeded. Patients from whom prospective specimens were not collected were asked to provide blood to blood cultures if a fungal infection occurred, even though specimens would postdate the onset of the fungal infection.

BMT recipients without clinical, radiographic, biopsy, or culture evidence of fungal infection were defined as patients with “no infection.” Recipients in whom disease proceeded. Patients from whom prospective specimens were not obtained on a weekly basis to determine colonization status. Blood cultures were performed with the BacT/Alert FAN aerobic culture bottle (Organon Teknika, Durham, N.C.) in combination with the Isolator aerobic culture tube (Wampole, Cranbury, N.J.). To obtain specimens from the days preceding in-
fec tion, patients were asked to donate 6 ml of whole-blood specimens prospect-
tively on a weekly basis. If the patient showed symptoms or signs relating to fungal disease, we attempted to collect specimens daily while the clinical workup for disease proceeded. Patients from whom prospective specimens were not collected were asked to provide blood to blood cultures if a fungal infection occurred, even though specimens would postdate the onset of the fungal infection.

BMT recipients without clinical, radiographic, biopsy, or culture evidence of fungal infection were defined as patients with “no infection.” Recipients in whom disease proceeded. Patients from whom prospective specimens were not obtained on a weekly basis to determine colonization status. Blood cultures were performed with the BacT/Alert FAN aerobic culture bottle (Organon Teknika, Durham, N.C.) in combination with the Isolator aerobic culture tube (Wampole, Cranbury, N.J.). To obtain specimens from the days preceding in-
fec tion, patients were asked to donate 6 ml of whole-blood specimens prospect-
tively on a weekly basis. If the patient showed symptoms or signs relating to fungal disease, we attempted to collect specimens daily while the clinical workup for disease proceeded. Patients from whom prospective specimens were not collected were asked to provide blood to blood cultures if a fungal infection occurred, even though specimens would postdate the onset of the fungal infection.

BMT recipients without clinical, radiographic, biopsy, or culture evidence of fungal infection were defined as patients with “no infection.” Recipients in whom disease proceeded. Patients from whom prospective specimens were not obtained on a weekly basis to determine colonization status. Blood cultures were performed with the BacT/Alert FAN aerobic culture bottle (Organon Teknika, Durham, N.C.) in combination with the Isolator aerobic culture tube (Wampole, Cranbury, N.J.). To obtain specimens from the days preceding in-
fec tion, patients were asked to donate 6 ml of whole-blood specimens prospect-
tively on a weekly basis. If the patient showed symptoms or signs relating to fungal disease, we attempted to collect specimens daily while the clinical workup for disease proceeded. Patients from whom prospective specimens were not collected were asked to provide blood to blood cultures if a fungal infection occurred, even though specimens would postdate the onset of the fungal infection.

BMT recipients without clinical, radiographic, biopsy, or culture evidence of fungal infection were defined as patients with “no infection.” Recipients in whom disease proceeded. Patients from whom prospective specimens were not obtained on a weekly basis to determine colonization status. Blood cultures were performed with the BacT/Alert FAN aerobic culture bottle (Organon Teknika, Durham, N.C.) in combination with the Isolator aerobic culture tube (Wampole, Cranbury, N.J.). To obtain specimens from the days preceding in-
fec tion, patients were asked to donate 6 ml of whole-blood specimens prospect-
tively on a weekly basis. If the patient showed symptoms or signs relating to fungal disease, we attempted to collect specimens daily while the clinical workup for disease proceeded. Patients from whom prospective specimens were not collected were asked to provide blood to blood cultures if a fungal infection occurred, even though specimens would postdate the onset of the fungal infection.

BMT recipients without clinical, radiographic, biopsy, or culture evidence of fungal infection were defined as patients with “no infection.” Recipients in whom disease proceeded. Patients from whom prospective specimens were not obtained on a weekly basis to determine colonization status. Blood cultures were performed with the BacT/Alert FAN aerobic culture bottle (Organon Teknika, Durham, N.C.) in combination with the Isolator aerobic culture tube (Wampole, Cranbury, N.J.). To obtain specimens from the days preceding in-
fec tion, patients were asked to donate 6 ml of whole-blood specimens prospect-
tively on a weekly basis. If the patient showed symptoms or signs relating to fungal disease, we attempted to collect specimens daily while the clinical workup for disease proceeded. Patients from whom prospective specimens were not collected were asked to provide blood to blood cultures if a fungal infection occurred, even though specimens would postdate the onset of the fungal infection.

BMT recipients without clinical, radiographic, biopsy, or culture evidence of fungal infection were defined as patients with “no infection.” Recipients in whom disease proceeded. Patients from whom prospective specimens were not obtained on a weekly basis to determine colonization status. Blood cultures were performed with the BacT/Alert FAN aerobic culture bottle (Organon Teknika, Durham, N.C.) in combination with the Isolator aerobic culture tube (Wampole, Cranbury, N.J.). To obtain specimens from the days preceding in-
fec tion, patients were asked to donate 6 ml of whole-blood specimens prospect-
tively on a weekly basis. If the patient showed symptoms or signs relating to fungal disease, we attempted to collect specimens daily while the clinical workup for disease proceeded. Patients from whom prospective specimens were not collected were asked to provide blood to blood cultures if a fungal infection occurred, even though specimens would postdate the onset of the fungal infection.

BMT recipients without clinical, radiographic, biopsy, or culture evidence of fungal infection were defined as patients with “no infection.” Recipients in whom disease proceeded. Patients from whom prospective specimens were not obtained on a weekly basis to determine colonization status. Blood cultures were performed with the BacT/Alert FAN aerobic culture bottle (Organon Teknika, Durham, N.C.) in combination with the Isolator aerobic culture tube (Wampole, Cranbury, N.J.). To obtain specimens from the days preceding in-
fec tion, patients were asked to donate 6 ml of whole-blood specimens prospect-
tively on a weekly basis. If the patient showed symptoms or signs relating to fungal disease, we attempted to collect specimens daily while the clinical workup for disease proceeded. Patients from whom prospective specimens were not collected were asked to provide blood to blood cultures if a fungal infection occurred, even though specimens would postdate the onset of the fungal infection.

BMT recipients without clinical, radiographic, biopsy, or culture evidence of fungal infection were defined as patients with “no infection.” Recipients in whom disease proceeded. Patients from whom prospective specimens were not obtained on a weekly basis to determine colonization status. Blood cultures were performed with the BacT/Alert FAN aerobic culture bottle (Organon Teknika, Durham, N.C.) in combination with the Isolator aerobic culture tube (Wampole, Cranbury, N.J.). To obtain specimens from the days preceding in-
fec tion, patients were asked to donate 6 ml of whole-blood specimens prospect-
tively on a weekly basis. If the patient showed symptoms or signs relating to fungal disease, we attempted to collect specimens daily while the clinical workup for disease proceeded. Patients from whom prospective specimens were not collected were asked to provide blood to blood cultures if a fungal infection occurred, even though specimens would postdate the onset of the fungal infection.

BMT recipients without clinical, radiographic, biopsy, or culture evidence of fungal infection were defined as patients with “no infection.” Recipients in whom disease proceeded. Patients from whom prospective specimens were not obtained on a weekly basis to determine colonization status. Blood cultures were performed with the BacT/Alert FAN aerobic culture bottle (Organon Teknika, Durham, N.C.) in combination with the Isolator aerobic culture tube (Wampole, Cranbury, N.J.). To obtain specimens from the days preceding in-
fec tion, patients were asked to donate 6 ml of whole-blood specimens prospect-
tively on a weekly basis. If the patient showed symptoms or signs relating to fungal disease, we attempted to collect specimens daily while the clinical workup for disease proceeded. Patients from whom prospective specimens were not collected were asked to provide blood to blood cultures if a fungal infection occurred, even though specimens would postdate the onset of the fungal infection.

BMT recipients without clinical, radiographic, biopsy, or culture evidence of fungal infection were defined as patients with “no infection.” Recipients in whom disease proceeded. Patients from whom prospective specimens were not obtained on a weekly basis to determine colonization status. Blood cultures were performed with the BacT/Alert FAN aerobic culture bottle (Organon Teknika, Durham, N.C.) in combination with the Isolator aerobic culture tube (Wampole, Cranbury, N.J.). To obtain specimens from the days preceding in-
fec tion, patients were asked to donate 6 ml of whole-blood specimens prospect-
tively on a weekly basis. If the patient showed symptoms or signs relating to fungal disease, we attempted to collect specimens daily while the clinical workup for disease proceeded. Patients from whom prospective specimens were not collected were asked to provide blood to blood cultures if a fungal infection occurred, even though specimens would postdate the onset of the fungal infection.

BMT recipients without clinical, radiographic, biopsy, or culture evidence of fungal infection were defined as patients with “no infection.” Recipients in whom disease proceeded. Patients from whom prospective specimens were not obtained on a weekly basis to determine colonization status. Blood cultures were performed with the BacT/Alert FAN aerobic culture bottle (Organon Teknika, Durham, N.C.) in combination with the Isolator aerobic culture tube (Wampole, Cranbury, N.J.). To obtain specimens from the days preceding in-
fec tion, patients were asked to donate 6 ml of whole-blood specimens prospect-
tively on a weekly basis. If the patient showed symptoms or signs relating to fungal disease, we attempted to collect specimens daily while the clinical workup for disease proceeded. Patients from whom prospective specimens were not collected were asked to provide blood to blood cultures if a fungal infection occurred, even though specimens would postdate the onset of the fungal infection.
Fonsecaea pedrosoi, Rhizomucor pusillus, and Sepedonium. DNA was believed to have been successfully extracted from each of these nonamplifiable organisms because DNA was visualized on a 2% agarose gel after electrophoresis and because amplification was successful for four of the organisms when a different set of panfungal primers was used (12). Specificity was confirmed by nonamplification of DNA from Staphylococcus aureus, viridans group streptococci, coagulase-negative staphylococci, Pseudomonas aeruginosa, and Escherichia coli and of human DNA (data not shown). The DNA of Prototheca wickerhamii, an alga, was amplified. Fungal organisms added to the ACD tubes containing blood can be detected after storage at 4°C for up to 18 months.

Results of the optimized PCR assay with human blood specimens. Specimens were collected from a total of ∼200 patients over a 1-year period. Forty blood and BMT recipients had no fungal disease or colonization, and multiple blood specimens from five of these patients, chosen as a convenience sample, demonstrated no PCR signal when they were tested in duplicate. For more than 100 patients, a yeast or mold was cultured from samples from superficial sites, but the patients were not thought to be invasively infected on clinical or other laboratory grounds. Specimens from these patients were not tested further. For 23 patients invasive fungal infections were documented by a positive culture result for a sample from a usually sterile site and/or by histopathologic appearance. Data for five patients were excluded because specimens were not collected at the point of infection. For six patients, the assay mixture was contaminated (sham controls were positive) during the testing of the patient’s specimens. Specimens from two patients with Aspergillus sinusits were not tested. We present the results for four patients with candidemia, three patients with pulmonary aspergillosis, three patients with mixed fungal infections, and one patient with fusariosis. For each of these 11 patients with documented invasive fungal infection, PCR assay of whole-blood specimens was positive at least once in the course of the patient’s illness.

Serial whole-blood specimens from the four candidemic patients generally showed positive PCR signals on the days that the blood cultures were positive (Fig. 1). Control blood samples from volunteer donors tested in the same assay runs as specimens from infected patients remained negative. C. parasitosis was repeatedly cultured from the blood of patient 10022 until the patient’s central venous catheter was removed. Patient 10701 developed C. glabrata fungemia just after the central venous catheter had been removed for persistent bacterial infection. For both patients, the blood culture was positive on 2 days when the PCR signal was negative, but this occurred toward the end of infection, after the patients had received significant amounts of antifungal therapy. Conversely, patient 11958, who also had C. parasitosis fungemia, had a negative PCR signal on the last day that the blood culture was positive but a positive PCR signal on the following day. Whether this signal represents a true-positive result due to the sensitivity of the assay or a false-positive result is not known. Few specimens from days which predated the candidemias by less than 1 week were available for testing, and specimens were not always obtained on days when blood samples for culture were drawn. Patient 10490 developed fungemia caused by C. tropicalis, C. albicans, and a Candida organism whose species was not determined, which led to a fungus-related death 18 days after the first blood sample was positive by culture. PCR signals were found on all days that blood cultures were positive when blood specimens were available, except for the day of the onset of infection. Several possible explanations for this false-negative signal include sampling error, prolonged cold storage time, transposition of specimen tubes, or suboptimal yield from the DNA extraction.

Specimens from three patients with pulmonary aspergillosis as the only fungal infection demonstrated occasional positive PCR results (Fig. 2). Two cultures of sputum from patient 9282 grew A. fumigatus, at which time a computed tomography (CT) scan of the chest did not reveal evidence of fungal disease. Two
weeks later, two of four specimens postdating a follow-up CT scan with fungal-like nodules were positive by PCR. The patient died of invasive pulmonary aspergillosis 3 weeks after the diagnostic CT scan. Patients 9886 and 10885 developed pulmonary aspergillosis while being treated with steroids in the outpatient setting. For both patients, PCR of whole blood became positive after the results of the diagnostic lung biopsy were already available, and the patients died within 2 weeks of the diagnosis of infection by biopsy.

The blood of three patients with mixed infections and one patient with fusariosis also had positive panfungal PCR signals (Fig. 3). For these patients the panfungal PCR assay could diagnose the presence of fungal DNA but could not suggest prospectively the presence of a mixed infection. Patient 10916 had a positive PCR assay result 3 days before demonstrating Candida fungemia and 5 days before the autopsy, which demonstrated pulmonary Aspergillus; in addition to persistent Candidemia or followed by the onset of aspergillosis. Patient 10419 had Pneumocystis carinii pneumonia, followed by the discovery of pulmonary aspergillosis at autopsy 2 weeks later. Three blood specimens had positive PCR signals prior to the demonstration of pulmonary aspergillosis at autopsy, but the PCR results were difficult to interpret in the setting of concurrent P. carinii pneumonia (P. carinii is an organism that is also theoretically detectable by the assay). The blood of patient 7872, who had fusariosis, had positive PCR signals on 5 of 10 days after the blood culture had turned negative. He died 12 days after the onset of the disseminated infection.

For patient 9937 two PCR signals could be interpreted as false-positive signals. This patient had chronic graft-versus-host disease and recurrent mucosal breakdown. While most of the patients described above served as their own controls and the specimens obtained weekly had negative PCR results until the days immediately surrounding the invasive fungal infection, patient 9937 was the only patient to demonstrate intermittent PCR signals positive for fungi before the definitive invasive infection which was found at autopsy. Among the specimens obtained weekly from this patient, 11 specimens were negative and 3 specimens were positive by PCR before his death. The first positive signal was on day 59 after transplantation, but a blood sample obtained on that day was negative by routine culture. He did have intermittent seeding of his blood by a variety of organisms, as demonstrated by blood cultures positive for a zygomycete, Klebsiella, and Serratia on day 64; the positive PCR result on day 59 could have been an early indication of the zygomycete. A gram-negative bacillus was cultured from the patient’s blood on day 74, Serratia was cultured on day 100, Prototheca was cultured on day 102, and Penicillium was cultured on day 106. C. glabrata and a nonviable mold (mycelia sterilia) were found in most autopsied organs on day 111. The two positive PCR signals which occurred on days 86 and 107, with a particularly strong signal for the specimen obtained on day 107 (4 days before what was in retrospect a fungus-related death), could have been false positive, could have been early indications of Prototheca or Penicillium infection, or could have represented transient fungemia not otherwise noted by blood cultures.

DISCUSSION

This report describes a novel panfungal PCR assay in a nonradioactive format which has a lower limit of detection of 4 CFU/ml of blood but which retains the ability to detect a broad range of fungi. The amplicon sequences predict that our probe will result in a panfungal assay. This prediction was confirmed by positive PCR results in tests with a myriad of organisms representative of many taxonomic genera. Specimens from BMT patients were chosen for testing by this assay because approximately 5% of these patients predictably develop fungal infections during the course of their transplant-associated neutropenia and other immunosuppression (1, 18, 49). However, the PCR method can also be applied to specimens from other patient populations, including immunocompromised patients with human immunodeficiency virus infection.

In order to screen for fungal infections in BMT patients at risk for fungal infection, an assay detecting multiple fungal genera with high sensitivity is needed. The genera which should be targeted by the primers include Candida, Aspergillus, and other less common but medically relevant fungi. Furthermore, the spectrum of organisms causing infection has changed from C. albicans to C. glabrata, Candida tropicalis, C. krusei, C. parapsilosis, and fluconazole-resistant strains of C. albicans since the introduction of fluconazole in 1992 as prophylaxis against fungal infections (17, 35, 36, 43, 50–52). A probe which is panfungal and nonradioactive can facilitate the screening process for detection of an unknown organism in a clinical diagnostic laboratory. Previous studies that used panfungal PCR assays are limited by the lack of a panfungal probe.
Six fungi did not react with the probe. We confirmed adequate cell wall lysis and DNA extraction by the presence of the usual smear of DNA when the DNA was run on an agarose gel. In most cases, we were able to confirm adequate preparation by performing amplification with another set of panfungal primers (12). Interestingly, not all organisms detected by the assay with another set of panfungal primers were detectable by the assay with our primers, and vice versa. At present there are no published sequence data for some of the organisms (mostly zygomycetes), so we are uncertain about the percentage homology with the primer sequences. In one case, the gel was negative and both PCR assays were negative, so the DNA may have not been adequately extracted, possibly because of a poorly lysed cell wall, or inhibitors unique to the fungus may have been present.

Animal model studies support the utility of PCR for the detection of disseminated fungal disease. They have demonstrated that PCR assays can be as sensitive as blood cultures for the detection of Candida yet specific enough to remain negative for colonized animals (24, 48). Kan (24) inoculated 20 mice with C. albicans intravenously and then sacrificed the mice in groups of five mice each at 1- to 4-day intervals. Blood culture and PCR results were 100% concordant. Candida was inoculated into a thigh muscle of 20 additional mice, which led to the formation of local abscesses. All 20 mice were negative by both blood culture and PCR of blood before and for up to 15 days after the abscesses were established. van Deventer et al. (48) inoculated 24 mice intravenously and then sacrificed the animals at 1, 6, 24, 48, 72, 96, or 144 h after inoculation. The PCR assay of blood was positive for 48 of 50 (96%) animals, while the blood culture was positive for 35 of 50 (70%) animals.

The results of previously published studies of PCR with patient blood specimens strengthen the results of these studies with animals. In one study in which Candida genus-specific primers were used, PCR signals were positive for 24 of 25 blood samples from 15 fungemic patients positive by culture (22). In another study which used panfungal PCR primers coupled to species-specific C. albicans or genus-specific Aspergillus hybridization probes, blood specimens tested PCR positive concurrently with positive blood cultures for four patients with candidemia and 0 to 8 days prior to the detection of pulmonary infiltrates in 13 patients with invasive Aspergillus (12). In that study, the rate of false positivity was 3%, because only 3 of 189 blood specimens from 100 subjects with febrile neutropenia (n = 29), fungal colonization (n = 36), or healthy volunteers (n = 35) were PCR positive. Thus, any detection of Aspergillus or Candida DNA in blood could be a harbinger of invasive disease, and Candida colonization does not appear to create false-positive PCR signals with blood specimens.

In our study, neither PCR nor blood culture proved to be uniformly more positive, and a negative blood culture on any given day did not necessarily imply that a patient was cured, because for our patients 11958, 10490, and 10916, days of positive cultures were interspersed with days of negative cultures. Conversely, patient 7872 with fusariosis had a PCR positivity rate of 50% when blood cultures were negative. The patient was receiving treatment against the organism during the 10-day sampling interval in question, and the treatment may have caused intermittent clearing of the organism from the bloodstream.

The possible false-negative PCR signals which occurred toward the end of the courses of candidemia for patients 11958 and 10701 could be the result of two types of sampling errors. The first is the specific time that the specimen for PCR was drawn from the patient in relation to the time that the blood sample for culture was drawn and when the daily dose of amphotericin B was administered. The specimen tubes were labeled only with the date on which the sample was drawn. The second possible reason is that the numbers of circulating organisms are lower toward the end of an infection. As the frequency of organisms approaches one detectable unit per assay, the assays will produce sporadic positive results, as delineated by the Poisson distribution. It is also possible that the lack of sensitivity of PCR can be attributed in part to the prolonged cold storage time in anticoagulant, an undetectable experimental error such as transposition of specimen tubes during the testing process, or the possibility that our procedure for lysing the organisms and extracting the DNA provided a suboptimal yield.

Contamination has been the main obstacle to the clinical application of PCR. Contamination occurs by airborne spore inoculation during the extraction process, by product carryover, and because of the presence of nonviable fungal spores found in reusable equipment, despite autoclaving. To minimize the risk of contamination by reusable equipment and maximize the fungal DNA yield from blood specimens containing small quantities of infecting fungi, we chose an enzymatic method of fungal cell wall disruption for the extraction procedure. β-1,3-Glucanase enzymes hydrolyze glucose polymers at β-1,3-glucan linkages in fungi, releasing laminaripentaose and resulting in fungal spheroplasts, which are modified organisms that have partially lost the cell wall and that have increased osmotic sensitivity (37). Until the 1970s, snail gut enzyme was the prototype enzyme used for fungal cell wall lysis, but the preparation had variable activity from batch to batch (26, 27). β-1,3-Glucanase products include zymolase (ICN Pharmaceuticals, Costa Mesa, Calif.), a natural β-1,3-glucanase purified from a submerged culture of Arthrobacter luteus in the fermentation of yeast (27), and lyticase (Sigma), a synthetic equivalent of β-1,3-glucanase (42). The use of lyticase avoids the impurities found in zymolase, such as β-1,3-glucanase, protease, mannanase, xylanase, phosphatase, and amylase.

We discarded the results for six patients (35%) due to contamination. To minimize the rate of contamination in the future, we will use the lyticase lysis enzyme, pre aliquoted reagents, dedicated pipettes, aerosol-resistant tips, separate rooms for DNA extraction, amplification, and PCR product analysis, and negative amplification controls placed intermittently among the samples in each amplification assay.

We assessed the feasibility of using our panfungal PCR assay with a limited series of whole-blood specimens (6 ml each) obtained from several BMT patients with and without documented fungal infections. The limitation of the assay for the candidemic patients was the lack of specimens from before the onset of fungemia that could be used to prove whether the PCR assay can demonstrate disease before culture can. For our candidemic patients, the PCR assay signal did not persist beyond the time that the signal was found by the traditional means of proving infection. Among the patients with invasive pulmonary aspergillosis, only patient 9282 had positive PCR results more than 1 week prior to the diagnosis of infection. Future experiments will be directed at improving the specimen collection schedule and storage procedure and improving the real-time testing of specimens before we proceed with a larger prospective trial.

We do not yet know whether the assay can distinguish between colonization and infection. We excluded the data for colonized patients because the interpretation of the results was...
beyond the scope of the present study. Investigation of colonized patients would be a worthwhile effort in future studies.

In summary, we have developed a panfungal PCR assay which has a lower limit of detection of 4 CFU/ml and which is applicable to a broad range of fungi. When following previously described methods to avoid contamination, the DNA extraction procedure runs smoothly, although unpredictable episodes of contamination have occurred in our laboratory. When applied to blood specimens from patients infected with fungi, the assay shows promise as a future diagnostic tool. Further studies are needed to define the sensitivity and specificity of this assay for the diagnosis of fungal infection prior to other clinical or laboratory indications of invasive fungal infection. The thrust of this study was to show that this PCR assay may be used as an adjunct to conventional methods for the detection of fungal infection or as a tool for describing the natural history of fungal infection. Our ultimate goal will be to evaluate the sensitivity and specificity of the assay results in predicting mold infection in larger numbers of serial specimens from immunosuppressed BMT patients.

ACKNOWLEDGMENTS

J.-A. van Burik is a recipient of a Clinician Scientist Award (K08 AI-014111) from the National Institute of Health. All authors are supported by grants CA-18029 and CA-15704 from the National Institutes of Health. The Biocomputing Shared Resource at Fred Hutchinson Cancer Research Center is supported by P30 CA-15704. We thank Pfizer Inc. (New York, New York) for an unrestricted research grant to support this study.

REFERENCES