General Primer-Mediated PCR for Detection of *Aspergillus* Species

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A PCR assay was developed for the diagnosis of invasive aspergillosis in immunocompromised patients. For this purpose, the complete nucleotide sequences of the genes encoding the 18S rRNA of *Aspergillus nidulans*, *Aspergillus terreus*, *Aspergillus niger*, and *Aspergillus flavus* were elucidated and aligned to the sequences of *Aspergillus fumigatus* and other clinically relevant prokaryotic and eukaryotic microorganisms. Genus-specific sequences could be identified in the V7 to V9 region of 18S rRNA. By using hot-start PCR, Southern blot hybridization, and restriction enzyme analysis, *Aspergillus*-specific and -sensitive determination was achieved. Five of six immunosuppressed mice experimentally infected with *A. fumigatus* developed infection, and rRNA could be detected in each case, even in livers with the absence of positive cultures. *Aspergillus* species were detected by PCR in four neutropenic patients with proven aspergillosis, although *Aspergillus* species had been isolated from only one bronchoalveolar lavage (BAL) fluid sample. *Aspergillus* species were detected by PCR in two more patients suspected of having infection. Positive PCR signals were obtained from the BAL samples of 3 of 8 neutropenic patients who had developed pulmonary infiltrates, but none were obtained from the samples of 14 nonimmunosuppressed patients. These results indicate the potential value of PCR to detect *Aspergillus* species in BAL samples and, therefore, to identify neutropenic patients at risk for invasive aspergillosis.

The genus *Aspergillus* is a ubiquitous saprophytic soil fungus which colonizes the respiratory tract in humans (6) and is responsible for opportunistic infection in immunocompromised patients (18, 21, 25). Indeed, invasive aspergillosis is responsible for up to 41% of the deaths of patients with acute leukemia, and despite the severity and high mortality attributable to this mycosis, there has been little progress in accurately diagnosing infection antemortem (4, 14). While isolation of the fungus from sputum and bronchoalveolar lavage (BAL) fluid is indicative of infection, both specimens lack sufficient specificity and sensitivity (9, 15). Although the invasive techniques necessary to obtain a biopsy are usually precluded because of profound thrombocytopenia, the histological demonstration of fungal elements in tissue is mandatory for proven infection (6). Serology has little to offer as an alternative since the immune response is desultory and, hitherto, the detection of antigen has met with little success. Since successful treatment depends upon intervening before fungal proliferation becomes overwhelming (1, 8) and since there is, as yet, no rapid reliable means of diagnosis, therapy is begun empirically, often on the most permissive basis such as persistent unexplained fever despite broad-spectrum antibacterial therapy, and continued until after the recovery of granulocytes.

Recent studies have described PCR methods for detecting *Aspergillus fumigatus* and *Aspergillus flavus* in clinical material from immunosuppressed patients (17, 20, 22). However, other species can be involved (6), and so it seemed important to us to ascertain that a patient had infection with any *Aspergillus* organism. We therefore elucidated the complete sequences of 18S rRNA of several species of *Aspergillus* and related fungi and aligned them with sequences from other sources. In this way, it was possible to develop a sensitive and specific detection assay for the genus *Aspergillus* which could be validated in

experimentally infected mice as well as in BAL fluid from patients who were at risk of pulmonary infection.

MATERIALS AND METHODS

Organisms and growth conditions. *A. fumigatus* (CBS113. 26), *A. flavus* (CBS108.30), *Aspergillus nidulans* (CBS100.20), *Aspergillus terreus* (CBS106.25), and *Aspergillus niger* (CBS102. 12) were obtained from the Centraalbureau voor Schimmelcultures (CBS; Baarn, The Netherlands). In addition, a panel of microorganisms was chosen to represent molds related to *Aspergillus* organisms, including two *Penicillium* strains, *Penicillium marneffei* (AZN 747) and *Penicillium chrysogenum* (AZN 1126), *Pseudallescheria boydii* (AZN 409), a *Fusarium* strain (AZN 441), *Paecilomyces variotii* (AZN 731), and *Rhizopus oryzae* (AZN 593); the yeasts *Candida albicans* (ATTC 90028), *Candida tropicalis* (AZN 393), *Candida krusei* (AZN 416), *Torulopsis* (*Candida*) *glabrata* (ATCC 90030), and *Cryptococcus neoformans* (ATCC 90112); as well as the bacteria *Streptococcus sanguis*, *Streptococcus mitis*, *Staphylococcus aureus* (ATCC 25923), *Pseudomonas aeruginosa* (ATCC 10145), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 10031), and *Enterobacter cloacae* (ATCC 13047). Clinical isolates were identified with standard methods and were assigned a University Hospital Nijmegen storage number (AZN number).

Sequence analysis of the 18S rRNA genes. Specific primers of *Aspergillus* organisms included the 18S rRNA sequence of *A. fumigatus* described by Barns and associates (2) and the 18S rRNAs of *A. flavus*, *A. nidulans*, *A. niger*, and *A. terreus* which were sequenced by us.

Mycelial growth was peeled off from the agar surface with sterile forceps and suspended in 600 μl of distilled water which had been pretreated with diethyl pyrocarbonate (DEPC). This suspension was added to 1 ml of 0.5-mm-diameter zirconium beads which had been washed and autoclaved in 0.2% sodium dodecyl sulfate (SDS) in 1 M sodium bicarbonate. Complete

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cell destruction was achieved by high-frequency shaking at 6,000 vibrations per min for 160 s in a Mini-Beadbeater system (Tecnolab Int., Alkmaar, The Netherlands). Next, the mixture was centrifuged for 15 min at 2,500 rpm (Eppendorf centrifuge). Nucleic acids were purified by successive phenol, phenol-chloroform-isoamyl alcohol (25:24:1), and chloroform-isoamyl alcohol extraction (13) followed by precipitation with ethanol overnight at $-20°C$ and were finally dissolved in 100 μl of DEPC-treated distilled water which contained 0.4 U of human placental RNase inhibitor per μl (RNasin; Promega, Leiden, The Netherlands).

The complete 18S rDNA was amplified by PCR using the general eukaryotic 5' and 3' 18S rRNA primers 5'-CCTGGT TGATCCTGCCAGTA-3' and 5'-GCTTGATCCTTCTGCA GGTT-3', respectively (7). PCR was performed with a 100-μl reaction mixture containing 10 mM Tris-HCl (pH 9.0 at 25°C), 10 mM KCl, 1.4 mM $MgCl_2$, 0.2 mM (each) deoxynucleoside triphosphates (dNTPs), 0.1% Triton X-100, 50 pmol of each of the two primers, 0.2 U of SuperTaq DNA polymerase (HT Biotechnology, Cambridge, England), and 0.5 μg of DNA from each *Aspergillus* isolate which had been denatured at 94°C for 5 min. Next, 30 cycles of amplification were performed by denaturing for 1 min at 94°C, annealing the primer for 1 min at 42°C, and allowing elongation for 3 min at 72°C. When successful, amplification generated a product of approximately 1,800 bp. Each product was analyzed by electrophoresis in 1.5% agarose gels. The amplification products required for sequencing were purified from low-melting-point agarose (LKB) by the Prep-a-Gene system (Bio-Rad Laboratories). DNA sequencing was performed by the dideoxy method (19). Annealing of a primer to the template was performed by heat denaturing double-stranded PCR products in the presence of a primer and then immediately freezing them in a CO_2-cooled ethanol bath. Primers selected from several conserved regions of 18S RNA served as sequencing primers. The resultant sequences were aligned to those of *A. fumigatus* and the other organisms detailed earlier by a sequence analysis software package (Genetics Computer Group at the University of Wisconsin) implemented on a VAX computer, and *Aspergillus* genus-specific sequences were selected and tested for specificity. Initially, it appeared impossible to distinguish *Aspergillus* organisms from the *Penicillium* and *Paecilomyces* strains. Consequently, the 18S rRNA genes of these fungi were also sequenced. Subsequent alignment resulted in two primers for the *Aspergillus* genus-specific PCR assay which yielded a 363-bp fragment for positive amplification: *Asp1*, 5'-CGGC CCTTAAATAGCCCGGTC-3', located in the V7 region; and *Asp2*, 5'-ACCCCCCTGAGCCAGTCCG-3', located in the V9 region of the 18S rRNA (see Fig. 1).

Experimentally infected mice. Ten CD-1 Swiss mice (Central Animal Laboratory, Nijmegen, The Netherlands) were immunosuppressed with 150 mg of cyclophosphamide (Endoxan-Asta) per kg of body weight given subcutaneously 3 days before and on the day of infection and protected from bacterial infection by daily intraperitoneal injections of 40 mg of imipenem-cilastatin (Merck, Sharpe & Dohme, Haarlem, The Netherlands) per kg. Six mice were infected intranasally with approximately 10^6 conidia of *A. fumigatus* under general anesthesia with 0.1 ml of 4.5% chloral hydrate per 10 g of body weight administered intraperitoneally while the other four served as controls. Treatment with imipenem was continued until 4 days after infection when the mice were killed and their lungs and livers were removed. The tissues were cut and divided into three portions. The first was used to inoculate Sabouraud glucose agar which was then incubated at 42°C for 5 days. A second portion was homogenized with disposable

pellet pestles and tubes (Kontes, Vineland, N.J.) in 600 μl of DEPC-treated distilled water, after which nucleic acids were obtained as described above. The remaining tissue was fixed in buffered formalin (4%), embedded in paraffin, and examined for the presence of fungus after *p*-aminosalicylic acid staining.

Clinical samples. BAL fluid samples were tested retrospectively by PCR. Eighteen samples of BAL fluid had been obtained from 14 neutropenic patients who had developed a variety of pulmonary infiltrates during neutropenia induced by treatment for hematological malignancy (Table 1). Aspergillosis was proven in four cases and suspected in two cases. As controls, 14 BAL fluid samples were obtained from patients who were unlikely to be at any risk for invasive aspergillosis. The BAL fluids were centrifuged at 2,500 rpm in an Eppendorf centrifuge for 15 min, and the supernatant was discarded. The residue was resuspended in a total volume of 600 μl of DEPC-treated water, and nucleic acids were isolated as described above.

Aspergillus **culture.** A pellet of BAL fluid was microscopically examined after fluorescent staining (Fungiqual; CIBA-GEIGY, Basel, Switzerland), plated on Sabouraud glucose (2%) agar, and cultured for 5 days at 28 and 42°C. Histopathological evidence of invasive aspergillosis was obtained from autopsy material.

Reverse transcription and hot-start PCR. rRNA was transcribed into cDNA at 37°C for 60 min in 20 μl of reaction mixture of 75 mM KCl, 50 mM Tris-HCl (pH 8.3), 6 mM $MgCl_2$, 10 mM dithiothreitol, 0.2 mM dNTPs (Boehringer GmbH, Mannheim, Germany), 50 pmol of the downstream primer *Asp2* (see above), 5 U of avian myeloblastosis virus reverse transcriptase (Promega), and 1 μg of nucleic acid (determined on a GeneQuant apparatus from Bio-Rad). One waxgem (AmpliWax; Perkin-Elmer Cetus, Leiden, The Netherlands) was added to the sample for PCR analysis, heated to 80°C to allow the wax to melt and to cover the surface, and then returned to room temperature to allow the wax to solidify. This prevents the nucleic acids from melting into the PCR mixture and allows the amplification reaction to start at a high temperature, thereby preventing premature annealing and extension of the primer. Eighty microliters of the following PCR mixture was then deposited on the surface of the wax: 50 mM KCl, 10 mM Tris-HCl (pH 8.9), 1.0 mM $MgCl_2$, 0.2 mM (each) dNTPs, 0.1% Triton X-100, 80 pmol of the upstream primer *Asp1* (see above), 40 pmol of the downstream primer *Asp2*, and 0.2 U of SuperTaq DNA polymerase (HT Biotechnology Ltd.). RNA-cDNA hybrids were denatured at 94°C for 5 min and then underwent 40 cycles consisting of denaturation at 94°C, primer annealing at 62°C, and elongation 72°C, each lasting 1 min. The recommendations of Kwok and Higuchi (12) were followed, and each technical step was kept strictly isolated to prevent contamination.

Analysis of amplified DNA. Aliquots of 25-μl samples were analyzed by electrophoresis on a 1.5% agarose gel stained with ethidium bromide (13). Before Southern blotting, the agarose gel was depurinated in 0.25 N HCl, denatured in 0.5 N NaOH–1.5 M NaCl, and transferred to a nylon membrane (Hybond; Amersham Int., Bucks, England) by diffusion blotting in 0.5 N NaOH–1.5 M NaCl. DNA was covalently bound to the membrane by baking at 80°C for 2 h. Membranes were then prehybridized in 6× SSC (1× SSC consists of 15 mM sodium citrate and 150 mM sodium chloride)–5× Denhardt solution (1× Denhardt solution consists of 0.02% polyvinylpyrrolidone, 0.02% Ficoll, and 0.02% bovine serum albumin)–0.1% SDS–250 μg of denatured sonicated herring sperm DNA per ml at 42°C for 2 h. Hybridization was achieved over 16 h at 42°C in 6× SSC–1× Denhardt solution–0.1% SDS–100 μg of

TABLE 1. Characteristics of hematological patients at risk for invasive aspergillosis

Patient	Sex, age	Underlying disease and/or condition[a]	Aspergillus infection	BAL culture	Other diagnostic procedure(s)	PCR	Granulocyte count (10^9/liter)	Antifungal i.v. therapy[b] (total dose)	Outcome
1	♂, 38	CML, BMT	Proven	Aspergillus species	Invasive aspergillosis at autopsy	+	<0.1	Amphotericin B (660 mg)	Deceased
2	♀, 51	MDS	Proven	Corynebacterium jeikeium	Disseminated aspergillosis in cerebrum and lungs; Aspergillus antigen in serum positive	+	<0.1	Amphotericin B (1,006 mg), then itraconazole (2,000 mg)	Deceased
3	♀, 42	ALL, BMT	Proven	Negative	Aspergillus hyphae in open-lung biopsy	+	0.1	Amphotericin B (196 mg), then itraconazole (800 mg)	Survived
4	♀, 33	AML	Proven	Candida albicans	None	+	<0.1	Amphotericin B (496 mg)	Survived
5	♀, 55	AML	Probable	Herpes simplex virus		+	<0.1	Amphotericin B (286 mg)	Survived
6	♂, 45	NHL	Probable	Negative		+	<0.1	Liposomal Amphotericin B (12,600 mg)	Survived
7	♀, 61	AML	Possible	Candida albicans		+	<0.1	Fluconazole (6,000 mg) and amphotericin B (1,206 mg)	Deceased
8	♂, 44	BMT, prednisone, azathioprine, GVHD	Possible	Penicillium species, Candida albicans, influenza virus type A		+	5.0	Amphotericin B (550 mg), then itraconazole (146,000 mg)	Survived
9	♂, 22	ALL	Unlikely	Negative		+	<0.1	Amphotericin B (463 mg), then itraconazole	Survived
10	♀, 55	CML	None	Pseudomonas aeruginosa		−	2.0		Survived
11	♂, 41	AML	None	Candida albicans		−	<0.1	Amphotericin B (366 mg)	Survived
12	♀, 60	AML	None	Herpes simplex virus		−	<0.1	Amphotericin B (196 mg)	Survived
13	♀, 32	ALL, BMT	None	Negative		−	<0.1		Survived
14	♀, 60	NHL	None	Negative		−	<0.1	Amphotericin B (70 mg)	Deceased

[a] CML, chronic myeloid leukemia; AML, acute myelogenous leukemia; ALL, acute nonlymphocytic leukemia; BMT, bone marrow transplant; NHL, non-Hodgkin lymphoma; MDS, myelodysplastic syndrome; GVHD, graft versus host disease.
[b] i.v., intravenous.

herring sperm DNA per ml–10^6 cpm of a ^{32}P-5′-end-labelled Aspergillus-specific oligonucleotide probe (Asp-p, 5′-ATGGA AGTGCGCGGCAATAAC-3′) per ml. The blots were washed twice at 42°C in 2× SSC–0.1% SDS and once in 0.5× SSC–0.1% SDS at 55°C, each for 30 min. Kodak Royal X-Omat films were exposed to the blots for 4 to 8 h between intensifying screens (Dupont) at −80°C to allow autoradiography.

Nucleotide sequence accession numbers. The nucleotide sequence data reported in this paper will appear in the EMBL, GenBank, and DDBJ nucleotide sequence data bases under the accession numbers X78537 (A. flavus), X78538 (A. niger), X78539 (A. nidulans), and X78540 (A. terreus).

RESULTS

Selection of an Aspergillus genus-specific primer set and probe. The sequence alignment data of the V7 to V9 regions of the rRNA of the different Aspergillus species showed extensive homology with those of Penicillium marneffei, Penicillium chrysogenum, and Paecilomyces variotii (Fig. 1) but not with those of the other microorganisms. One primer which was fully homologous to all Aspergillus species but had a single mismatch to Penicillium chrysogenum, Paecilomyces variotii, and Penicillium marneffei (Fig. 1) was selected from the variable region V7. The other primer selected from the variable region V9 was not absolutely conserved for all Aspergillus species and displayed one or two mismatches and dispersed variability. Peni-

cillium marneffei contained only one mismatch while the Paecilomyces isolate was completely homologous (Fig. 1). Penicillium chrysogenum contained four mismatches, making it unsuitable for hot-start PCR with high-temperature annealing.

The probe finally selected comprised part of the conserved region between the variable regions V7 and V8 and was homologous to the Aspergillus isolates and Paecilomyces variotii but not Penicillium marneffei, in which the sequence contained two mismatches. A single base difference in the variable region V8 between the Aspergillus species and the other molds yielded an endonuclease StyI site, thereby permitting endonuclease restriction analysis of the amplification products to distinguish the Aspergillus species from Paecilomyces variotii (Fig. 1). Thus, by using a PCR assay that combines amplification, hybridization, and endonuclease restriction analysis, it should be possible to specifically identify Aspergillus species.

Specificity of the Aspergillus genus-specific PCR assay. Hot-start PCR was performed to exclude nonspecific primer annealing. At an annealing temperature of 62°C, specific amplification was observed with all the Aspergillus species and also with Penicillium marneffei and Paecilomyces variotii but with none of the other microorganisms, including Penicillium chrysogenum (Fig. 2). Southern blot analysis with the internal oligonucleotide probe showed clear hybridization with all the Aspergillus species and Paecilomyces variotii but not with Penicillium marneffei (Fig. 2). To distinguish Aspergillus spp. from Paecilomyces variotii, 25 μl of each amplification reaction was digested with StyI (Fig. 3) and the amplification product of 363

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                      Asp1                V7
 1: GATAACGAAC GAGACCTCGG CCCTTAAATA GCCCGGTCCG CATTTGCGGG CCGCTGGCTT
 2: .......... .......... .......... .......... .G.CC..... ..........
 3: .......... .......... .......... .......... .G........ -.........
 4: .......... .......... .......... .......... .G........ ..........
 5: .......... .......... .......... .......... .......... ..........
 6: .......... ........C. .......... .......... .G........ .......T..
 7: .......... ........C. .......... .......... .......... ..........
 8: .......... ........C. .......... .......... .G........ ......N...

                                        Asp-p
 1: CTTAGGGGGA CTATCGGCTC AAGCCGATGG AAGTGCGCGG CAATAACAGG TCTGTGATGC
 2: .......... ......C... .......... .......... .......... ..........
 3: .......... .......... .......... .......... .......... ..........
 4: .......... .......... .......... .......... .......... ..........
 5: .......... .......... .......... .......... .......... ..........
 6: .......... .......... .......... .......... .......... ..........
 7: .....A.... .......... .......... .......... .......... ..........
 8: .......... .......... .......... ....A..T.. .......... ..........

                                                         StyI
 1: CCTTAGATGT TCTGGGCCGC ACGCGCGCTA CACTGACAGG GCCAGCGAGT ACATCACCTT
 2: .......... .......... .......... .......... .G........ ..........
 3: .......... .......... .......... .......... .......... ..........
 4: .......... .......... .......... .......... .......... ..........
 5: .......... .......... .......... .......... .......... ..........
 6: .......... .......... .......... .......... .......... ..........
 7: .......... ........G. .......... .......... .......... ..........
 8: .......... ......G... .......... .......... .......... ..........
                           V8
 1: GG-CCGAGAG GTCTGGGTAA TCTTGTTAAA CCCTGTCGTG CTGGGGATAG AGCATTGCAA
 2: ..G....... .C.C...... .......... .......... .......... ..........
 3: .......... .......... .......... .......... .......... ..........
 4: ..C....... ...C...... -......... .......... .......... ..........
 5: .......... .......... .......... .......... .......... ..........
 6: .A........ .......... .......... .......... .......... ..........
 7: AA........ ..T....... .......... .......... .......... ..........
 8: .......... .......... .......... .......... .......... ..........

 1: TTATTGCTCT TCAACGAGGA ATGCCTAGTA GGCACGAGTC ATCAGCTCGT GCCGATTACG
 2: .......... .......... .......... .......... .......GC. ..........
 3: .......... .......... .......... .......... .......... ..........
 4: .......... .......... .......... .......... .......... ..........
 5: .......... .......... .......... .C........ .......... ..........
 6: .......... .......... ...T...... .......... .......... ..........
 7: .......... .......... .......... .......... .......... ..........
 8: .......... .......... .......... .......... .......... ..........

 1: TCCCTGCCCT TTGTACACAC CGCCCGTCGC TACTACCGAT TGAATGGCTC GGTGAGGCCT
 2: .......... .......... ....T..... .......... .......... .A........
 3: .......... .......... .......... .......... .......... ..........
 4: .......... .......... .......... .......... .......... .......-..
 5: .......... .......... .......... .......... .......... ..........
 6: .......... .......... .......... .......... .......... A.........
 7: .......... .......... .......... .......... .......... .A........
 8: .......... .......... .......... .......... .......... A.........

               Asp2                    V9
 1: TCGGACTGGC TCAGGGGAGT TGGCAACGAC TCCCCAGAGC CGGAAAGTTG GTCAAACCCG
 2: C......... .....A.G.. .......... C......... ......C.. ..T.......
 3: .......... .....A.G.. .......... C......... .......... ..........
 4: .......... C....A.G.. .......... C........G .......... ..........
 5: .......... ..CA...G.. .......... .......... .......... ..........
 6: .G....G... .....G.... .......... C......... .......... .......T..
 7: .G....T.... .T...A.G.. .......... C......... .......... .......T..
 8: .......... ....A..G.. .......... CG.T...... .......... .......TT.
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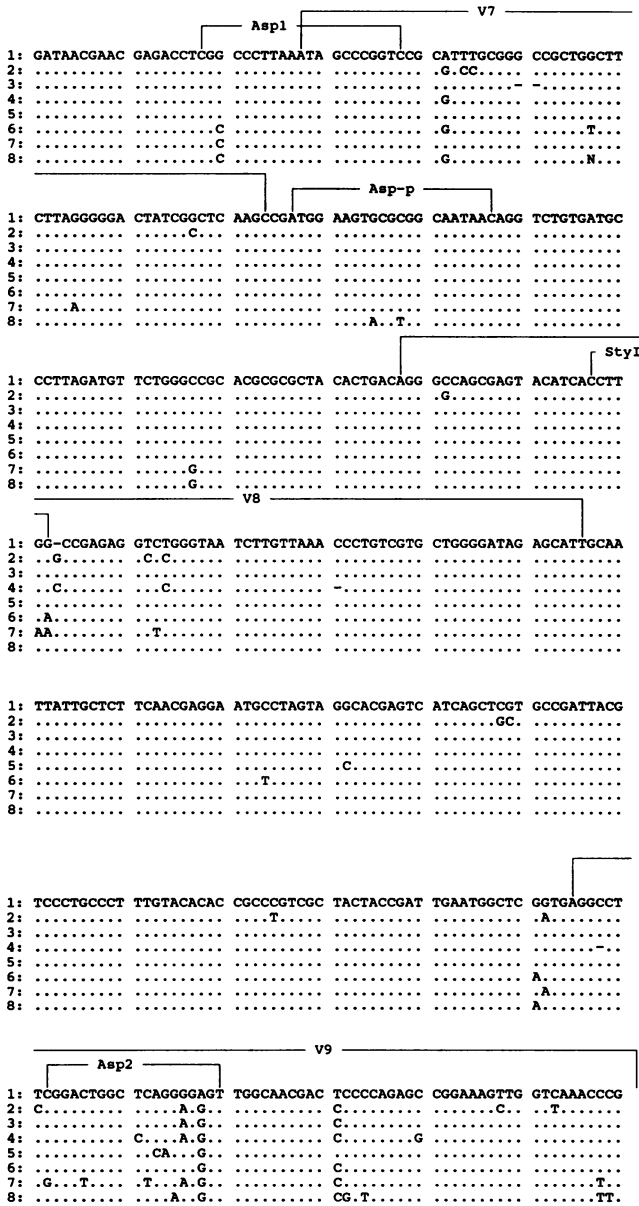
FIG. 1. Sequence alignment and primer selection for *Aspergillus* genus-specific PCR assay. Lanes: 1, *A. fumigatus*; 2, *A. nidulans*; 3, *A. niger*; 4, *A. flavus*; 5, *A. terreus*; 6, *Paecilomyces variotii*; 7, *Penicillium chrysogenum*; 8, *Penicillium marneffei*. *Asp1*, *Asp2*, and *Asp-p* represent the regions of the *Aspergillus* primers and probe (for exact sequences, see Materials and Methods). Above the sequences, the variable regions V7 to V9 are indicated. *Sty*I, the *Sty*I endonuclease restriction site, CCT(T/A)GG.

bp found in each *Aspergillus* species was digested in two fragments of 160 and 203 bp, whereas the PCR product of *Paecilomyces variotii* remained intact.

Sensitivity of the *Aspergillus* genus-specific PCR. Serial 10-fold dilutions of purified *A. fumigatus* nucleic acids isolated from the same sample were tested by PCR. Without prior transcription of the rRNA, a sensitivity of 1 pg of nucleic acid was obtained (on the basis of the DNA content), as detected by gel electrophoresis (Fig. 4A). However, when the rRNA was first transcribed into cDNA, 10 fg of nucleic acids was detected

on the gel (Fig. 4B). Southern blot analysis with the *Aspergillus* oligonucleotide probe increased the sensitivity another 10-fold.

Experimentally infected mice. *A. fumigatus* was cultured from multiple foci in the lungs of all six infected mice (mice 1 to 6, Table 2) while the PCR was positive for only five animals (Fig. 5). Hyphae were also found in the livers, although *A. fumigatus* was not recovered by culture. PCR on liver tissue was positive in each case, although the amplification signal was less intense than that obtained from the corresponding lungs (Fig. 5). In mouse 2, there was no histological evidence for invasive aspergillosis in either the lung or liver, and the culture and PCR both proved negative. There was neither histological nor cultural evidence of *A. fumigatus* in any of the lungs and livers from the healthy, immunosuppressed control mice (mice 7 to 10, Table 2), and the PCR was negative in every case.

Clinical samples. Positive amplification was obtained from nine patients, four of whom had proven cases and two had suspected cases of aspergillosis. An unspeciated *Aspergillus* strain had been isolated from one BAL fluid sample from which a strong amplification signal was also obtained (patient 1; Fig. 6). This patient was one of two who succumbed to invasive aspergillosis. The other patient (patient 2) died of disseminated aspergillosis, and only *Corynebacterium jeikeium* had been isolated from BAL fluid, although serum samples had been repeatedly positive for an *Aspergillus* antigen (Pastorex *Aspergillus*; Sanofi Pasteur, Paris, France). Hyphae consistent with *Aspergillus* species were seen in lung biopsy material obtained from patient 3, who had undergone open-lung surgery to exclude aspergillosis. Culture of the biopsy remained negative, but PCR was positive for *Aspergillus* species (Fig. 6, lanes 4). Three months later, this patient underwent BAL, having developed a pulmonary infiltrate during another episode of neutropenia. Cultures of BAL fluid were again negative, but PCR remained positive (Fig. 6, lanes 5). The last patient with proven aspergillosis (patient 4) had initially developed a pulmonary infiltrate 8 months earlier during remission induction therapy. *Candida albicans* was isolated from the BAL fluid sample, although *A. fumigatus* had been isolated from oral cultures. Then, during a second course of chemotherapy, a cavity developed in the right upper lobe consistent with lesions of invasive aspergillosis but culture of the BAL fluid was again negative. Both BAL fluid samples were positive by PCR for *Aspergillus* species. Patient 5 survived a pulmonary infiltrate suspected to be due to aspergillosis. Herpes simplex virus was isolated from the BAL fluid, and only 286 mg of amphotericin B was given as therapy when neutrophils recovered. BAL fluid from patient 6 was negative in culture, and he survived after being treated with 4 mg of liposomal amphotericin B (Ambisome; Vestar Europe) per kg for 36 days. *Candida albicans* was both seen microscopically and isolated from the BAL fluid of patient 7, who ultimately died of her underlying disease. However, no autopsy was performed to exclude invasive aspergillosis. She had been treated with more than 1 g of amphotericin B and had also received fluconazole (400 mg/day) for 15 days. Patient 8 was suffering from severe graft host disease after an allogeneic bone marrow transplant. Two BAL fluid samples were available for PCR analysis. The first BAL fluid sample had been obtained 20 months after transplant when the patient had developed an infiltrate in the right lower lobe. An unspeciated *Penicillium* isolate and *Candida albicans* were cultured. The patient received a short course of amphotericin B and was given 400 mg of itraconazole per day as a prophylaxis during the next 8 months. A second BAL fluid sample was obtained when no infiltrates were apparent on a chest X ray. This time, a *Penicillium* species together with influenza virus type A was isolated. However, both BAL fluid
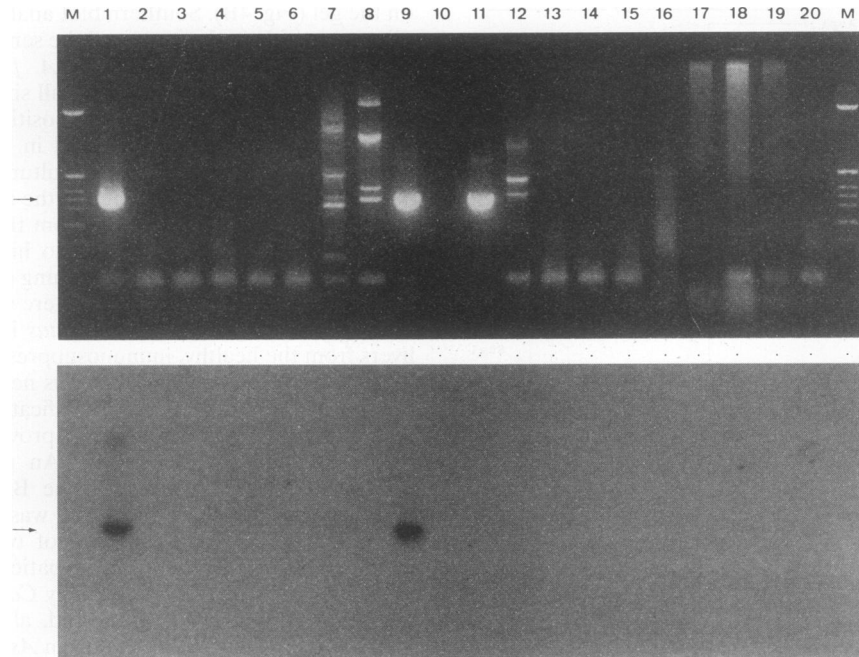
FIG. 2. Specificity of the *Aspergillus* genus-specific PCR. PCR with the *Aspergillus* genus-specific primer set was performed on a number of fungal and bacterial species. The reactions were analyzed by agarose gel electrophoresis (top) and Southern blot hybridization with a γ-^{32}P-labelled oligonucleotide probe (*Asp-p*) (bottom). Lanes: M, size marker (*Hin*fI-digested pBR322); 1, *A. fumigatus*; 2, *Candida albicans*; 3, *Candida tropicalis*; 4, *Candida krusei*; 5, *Torulopsis* (*Candida*) *glabrata*; 6, *Pseudallescheria boydii*; 7, a *Fusarium* strain; 8, *Enterobacter cloacae*; 9, *Paecilomyces variotii*; 10, *Penicillium chrysogenum*; 11, *Penicillium marneffei*; 12, *Cryptococcus neoformans*; 13, a *Rhizopus* strain; 14, *Streptococcus sanguis*; 15, *Streptococcus mitis*; 16, *Staphylococcus aureus*; 17, *Pseudomonas aeruginosa*; 18, *Escherichia coli*; 19, *Klebsiella pneumoniae*; 20, distilled water. Arrows indicate PCR fragment of 363 bp.

samples were positive by PCR (Fig. 6, lanes 2 and 3). The last patient (patient 9) from whom the BAL fluid sample was PCR positive had developed bilateral pulmonary infiltrates which resolved following neutrophil recovery while completing treatment for acute nonlymphocytic leukemia. All positive samples were confirmed to be *Aspergillus* specific by *Sty*I restriction endonuclease digestion. Samples from the 5 remaining pa-
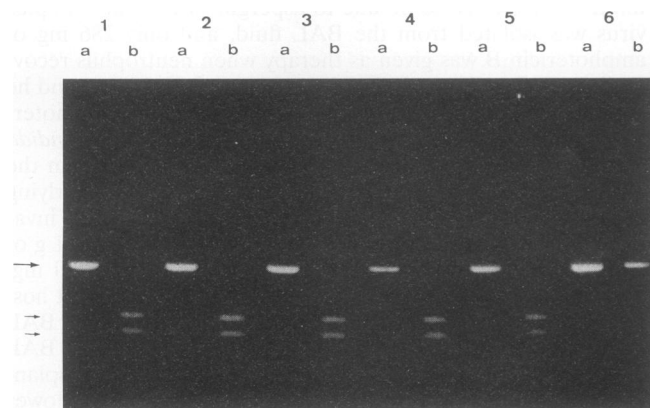

FIG. 3. Analysis of *Aspergillus* genus-specific PCR fragments. A sample of the PCR mixture was digested with *Sty*I. The restriction endonuclease reactions were analyzed by agarose gel electrophoresis. Lanes: 1, *A. fumigatus*; 2, *A. flavus*; 3, *A. terreus*; 4, *A. nidulans*; 5, *A. niger*; 6, *Paecilomyces variotii*. a, PCR control fragment of 363 bp (also indicated by large arrow). b, PCR fragments digested with *Sty*I (also indicated by small arrows at 160 and 203 bp, respectively).

tients were negative by PCR as were those obtained from 14 nonimmunosuppressed patients.

DISCUSSION

Invasive aspergillosis is one of the major clinical concerns in immunocompromised patients because it is difficult to detect, even in the case of extensive systematic infections, and treatment appears to be successful only when started in an early stage of disease (8). While prevention of infection remains the ultimate goal, identifying those individuals at greatest risk by a rapid diagnosis would provide an alternative since these are most likely to benefit from early treatment. Recently, several investigators reported the use of PCR to detect *A. fumigatus* and *A. flavus* in high-risk patients (17, 20, 22). However, infection due to other species is not uncommon (11, 25), and so it seems important to detect all clinically relevant *Aspergillus* species by developing a genus-specific PCR assay such as we describe.

rRNA has been used as the target for the development of species- or genus-specific PCR assays for several different microorganisms, such as mycobacteria and mycoplasmas (3, 24). Since only the 18S rRNA of *A. fumigatus* had been previously sequenced (2), we elucidated the complete 18S rRNA sequences of the other opportunistic pathogens *A. flavus*, *A. terreus*, *A. nidulans*, and *A. niger*. Alignment of the sequences revealed the impossibility of selecting *Aspergillus* species-specific primers for PCR. However, several domains, mainly in the variable regions V7 to V9, which appeared to be *Aspergillus* genus specific were identified. A suitable-looking primer pair was therefore selected from this region, but strong cross-hybridization was observed with *Paecilomyces variotii*,
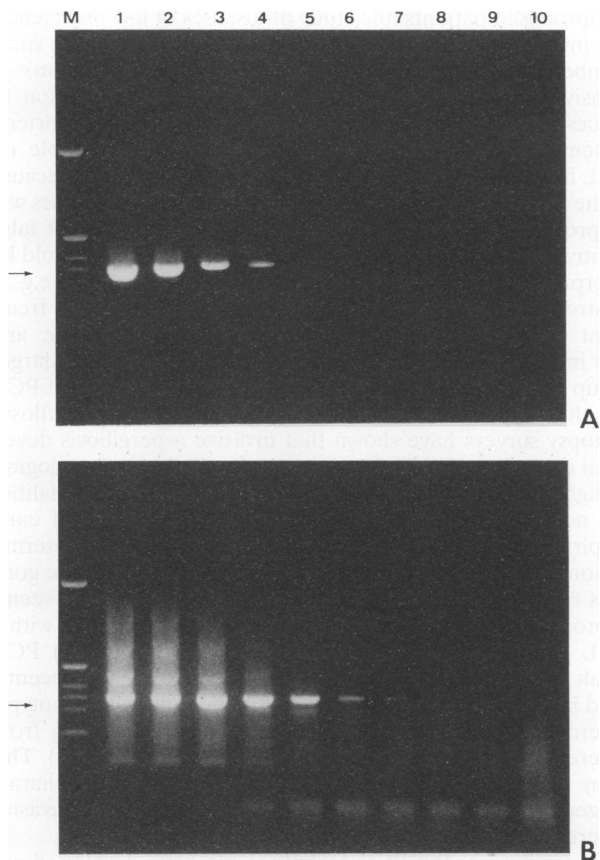
FIG. 4. Sensitivity of the detection of diluted purified nucleic acids isolated from *A. fumigatus* by PCR. The nucleic acid extract either was used directly in the PCR (DNA PCR) (A) or was first transcribed into cDNA by reverse transcription prior to the PCR (RNA/DNA PCR) (B). Lanes: M, size marker (*Hin*fI-digested pBR322); 1, 10 ng; 2, 1 ng; 3, 100 pg; 4, 10 pg; 5, 1 pg; 6, 100 fg; 7, 10 fg; 8, 1 fg; 9, 100 ag; 10, 10 ag. Arrows indicate PCR fragment of 363 bp.

TABLE 2. Detection of *A. fumigatus* in organs of experimentally infected mice

Mouse	Clinical sample	Detection by:		
		Culture	Histology	PCR
1	Lung	+	+	+
	Liver	−	+	+
2	Lung	+	−	−
	Liver	−	−	−
3	Lung	+	+	+
	Liver	−	+	+
4	Lung	+	+	+
	Liver	−	+	+
5	Lung	+	+	+
	Liver	−	+	+
6	Lung	+	+	+
	Liver	−	+	+
7	Lung	−	−	−
	Liver	−	−	−
8	Lung	−	−	−
	Liver	−	−	−
9	Lung	−	−	−
	Liver	−	−	−
10	Lung	−	−	−
	Liver	−	−	−

ples since a single genome of *A. nidulans* is estimated to contain approximately 50 fg of chromosomal DNA, based on the karyotype of *A. nidulans*, which is equivalent to about 20 fungal elements (5).

The potential value of the PCR was demonstrated in a mouse model in which PCR was positive in five of the six infected immunocompromised mice which had developed in-

Penicillium marneffei, and *Penicillium chrysogenum*. Spreadbury and colleagues (20) also found reactions suggesting a close homology between *Penicillium* and *Aspergillus* spp. based on the 26S rRNA sequence. Subsequent sequencing of the rRNAs of these fungi showed a close homology to exist among the *Aspergillus* species. A primer pair from the V7 and V9 region was selected (Fig. 1), and it was possible to differentiate *Aspergillus* species from these three closely related genera by combining hot-start PCR with high annealing temperature, hybridization with a specific oligonucleotide probe, and restriction endonuclease digestion with *Sty*I. However, it cannot be excluded that other *Penicillium* or *Paecilomyces* species or, in fact, other related genera that might be contaminants can be reactive in the assay. On the other hand, no specific cross-hybridization was observed with any other prokaryotic or eukaryotic microorganism. Without prior transcription of the rRNA, 1 pg of nucleic acids was detected on the gel. Sensitivity was increased to 10 fg when the rRNA was first transcribed into cDNA, and it was extended 10-fold by Southern blot hybridization. However, RNA amplification did not lead to the identification of more positive clinical samples than were found by rDNA amplification (data not shown), indicating that sufficient sensitivity was obtained without this additional step. Moreover, this provides sufficient sensitivity for clinical sam-
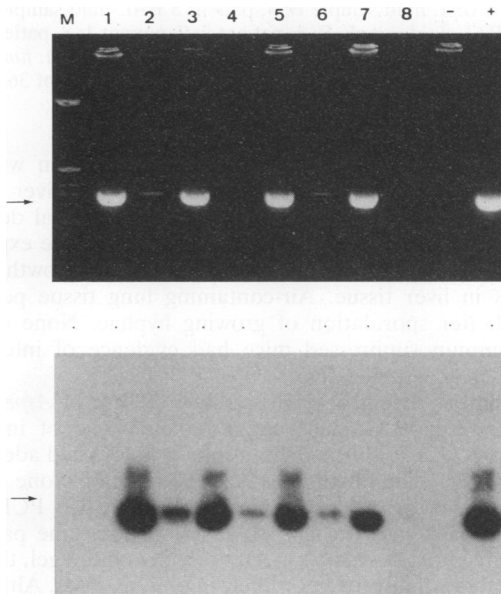

FIG. 5. PCR analysis of experimentally infected mice. Nucleic acids were extracted from the lungs and livers, subjected to PCR amplification, and analyzed by agarose gel electrophoresis (top) and Southern blot hybridization (bottom). Lanes: M, size marker (*Hin*fI-digested pBR322); 1, lung of mouse 1; 2, liver of mouse 1; 3, lung of mouse 2; 4, liver of mouse 2; 5, lung of mouse 3; 6, liver of mouse 3; 7, lung of mouse 4; 8, liver of mouse 4; −, lung of uninfected mouse; +, *A. fumigatus* DNA. Arrows indicate PCR fragment of 363 bp.
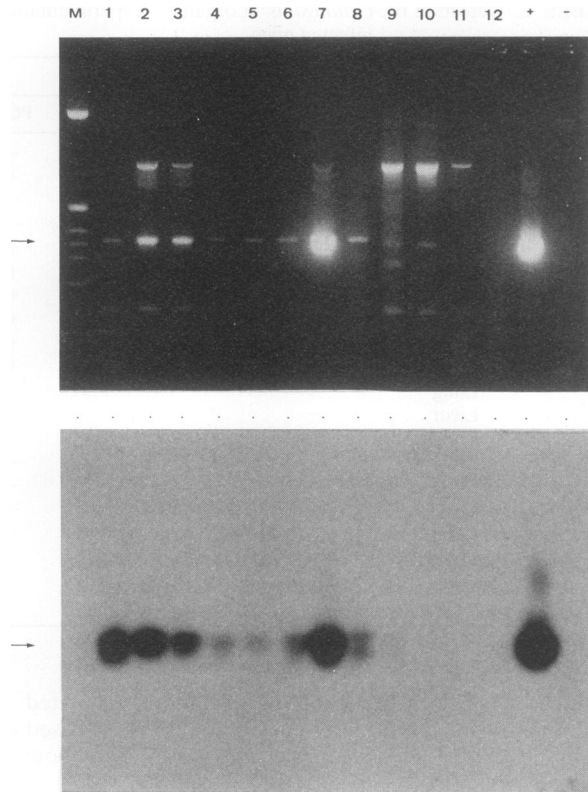
FIG. 6. PCR analysis of patients at risk for invasive aspergillosis. Nucleic acids were extracted from BAL fluid samples, subjected to PCR amplification, and analyzed by agarose gel electrophoresis (top) and Southern blot hybridization (bottom). Lanes: M, size marker (*Hin*fI-digested pBR322); 1, patient 7; 2, patient 8 BAL fluid sample A; 3, patient 8 BAL fluid sample B; 4, patient 3 BAL fluid sample A; 5, patient 3 BAL fluid sample B; 6, patient 2; 7, patient 1; 8, patient 4; 9 through 12, BAL fluid samples from control patients; +, *A. fumigatus* DNA; −, distilled water. Arrows indicate PCR fragment of 363 bp.

vasive aspergillosis following intranasal inoculation with *A. fumigatus*. In contrast to the lung specimens, the liver specimens revealed positive PCR signals and histological demonstrations of hyphae but negative cultures. A possible explanation could be the absence of conidia during the growth of *A. fumigatus* in liver tissue. Air-containing lung tissue possibly induces better sporulation of growing hyphae. None of the control immunosuppressed mice had evidence of infection, and each PCR was negative.

In a clinical setting, a single positive culture of *Aspergillus* organisms from BAL fluid might be indicative of invasive aspergillosis (15, 25) but only multiple samples yield adequate sensitivity (27). The fungus was isolated from only one of the four patients with invasive aspergillosis, although PCR was positive in each case. In fact, BAL fluids from nine patients were positive for *Aspergillus* species directly on the gel, though infection was either proven or likely in only six cases. Although it is possible that the other three patients with a positive PCR test result might have had incipient aspergillosis, bronchial colonization cannot be excluded (6). Additional Southern blot hybridization did not alter the number of patients with positive results, suggesting that no more than 9 of the 14 patients (64%) who developed pulmonary infiltrates during immunosuppression were actually at risk of aspergillosis. Although our tertiary-care 1,000-bed university hospital admits several immuno-

compromised patients, the study of diseases of low prevalence, like invasive aspergillosis, will inevitably be limited by the small number of patients. The only definitive diagnosis of proven invasive aspergillosis is by histopathological examination of tissues. In most instances, this is only possible postmortem. Antemortem diagnosis is, in our hospital, only possible on BAL fluid and, in most cases, not by open-lung biopsy because of the associated thrombocytopenia. Therefore, most cases will be probable or possible. There is always a possibility of false positivity with this approach. However, PCR results should be interpreted in the whole clinical context of the patient (e.g., a neutropenic or otherwise immunocompromised patient, treatment with broad-spectrum antibiotics, persisting fever, and new infiltrates on chest X ray). We have begun to study a larger group of patients to prospectively determine the value of PCR for the rapid and early diagnosis of invasive aspergillosis. Autopsy surveys have shown that invasive aspergillosis developed in around 45% of the patients who died of hematological malignancy (14, 26). However, current treatment modalities are not without risk, and even though the practice of early empiric therapy is fairly widespread (10, 16), a better determination of the group of patients at risk remains a desirable goal. This requires a test with maximum sensitivity, and so it seems appropriate to consider any immunosuppressed patient with a BAL fluid sample yielding a positive (Southern blot) PCR result as being at risk of invasive aspergillosis. We recently used interrepeat PCR fingerprinting to demonstrate genotypic heterogenicity among *A. fumigatus* isolates originating from different patients and different anatomical locations (23). This assay by which different isolates can be genotypically characterized can be used in the study of the epidemiology of invasive aspergillosis.

In conclusion, the PCR technique we have developed appears suitable to study a large group of patients and to identify those at risk of invasive aspergillosis. One of the main limitations now is that the method itself cannot yet be easily performed in every microbiological laboratory, since trained personnel and experience with PCR are necessary. However, once validated, a method such as ours could have a major impact in reducing the morbidity and mortality of patients undergoing treatment for cancer and hematological malignancy by allowing the early institution of therapy on a more selective basis than is possible at present.

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