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Development of a LightCycler PCR Assay for Detection and Quantification of *Aspergillus fumigatus* DNA in Clinical Samples from Neutropenic Patients

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The increasing incidence of invasive aspergillosis, a life-threatening infection in immunocompromised patients, emphasizes the need to improve the diagnostic tools for this disease. We established a LightCycler-based real-time PCR assay to detect and quantify rapidly, specifically, and sensitively *Aspergillus fumigatus* DNA in both bronchoalveolar lavage (BAL) and blood samples from high-risk patients. The primers and hybridization probes were derived from an *A. fumigatus*-specific sequence of the mitochondrial cytochrome *b* gene. The assay is linear in the range between 13.2 fg and 1.3 ng of *A. fumigatus* DNA, corresponding to 3 to 300,000 CFU per ml of BAL fluid or blood. No cross-amplification was observed with human DNA or with the DNA of fungal or bacterial pathogens. For clinical evaluation we investigated 10 BAL samples from nine neutropenic patients with malignant hematological diseases and 12 blood samples from seven neutropenic patients with malignant hematological diseases. Additionally, we tested one blood sample and one BAL sample from each of two neutropenic patients. In order to characterize the validity of the novel PCR assay, only samples that had shown positive results by a previously described sensitive and specific nested PCR assay were tested. Twelve of 12 BAL samples and 6 of 14 blood samples gave positive results by the LightCycler PCR assay. Eight of 14 blood samples gave negative results by the novel method. The LightCycler PCR-mediated quantification of the fungal burden showed 15 to 269,018 CFU per ml of BAL sample and 298 to 104,114 CFU per ml of blood sample. Twenty of 20 BAL samples and 50 of 50 blood samples from subjects without evidence of invasive pulmonary aspergillosis (IPA) were PCR negative. Compared to a previously described nested PCR assay, these preliminary data for the novel real-time PCR assay indicate a less sensitive rate of detection of IPA in high-risk patients, but the assay may be valuable for quantification of the fungal burden in individual clinical samples.

The increasing incidence of life-threatening systemic fungal infections emphasizes the need to improve the presently limited diagnostic tools for the detection and monitoring of these infections (46). The highest risk occurs during induction of treatment for acute leukemia or after bone marrow transplantation, leading to prolonged periods of neutropenia (5, 6, 12–15, 20, 38, 40, 45, 50, 51). Invasive infections with *Aspergillus* species in particular are increasing, resulting in high mortality rates or causing high morbidity rates (33). Delayed diagnosis and therapy of invasive pulmonary aspergillosis (IPA) worsen the prognosis (50). Due to the poor prognosis, all diagnostic approaches primarily aim at an early confirmation of the infection (27, 48). Furthermore, these patients are at high risk of life-threatening relapses of IPA when they need to be retreated for their primary disease, particularly if an *Aspergillus* infection has not been previously diagnosed or treated adequately (51).

Until now, only limited diagnostic tools with poor sensitivities and reliabilities were available for early detection of invasive aspergillosis (15, 21, 52), with the systemic infection frequently being diagnosed late or confirmed only at autopsy (6, 20, 40, 43). At present, positive results solely from conventional cultures or histological examination provide definitive proof of invasive aspergillosis (2, 48), although tissue biopsy specimens are not generally available (15, 16, 19) and cultures of blood, bronchial, and bronchoalveolar lavage (BAL) samples are often negative. High-resolution computed tomography of the lung is accepted as a hint but not as proof of IPA (2, 10, 11). A novel serological enzyme-linked immunosorbent assay based on the detection of galactomannan showed high rates of sensitivity and specificity (9, 35), but additional appropriate imaging techniques are recommended to improve the diagnosis (26, 36).

Sensitive and rapid molecular detection assays have been established by use of the PCR method. Previous studies evaluating PCR-mediated detection of *Aspergillus* species showed significantly high rates of sensitivity but involved assays with different methods and objectives, partly for optimization of

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culture assays (37) and partly for typing in epidemiological studies (3, 4).

In order to achieve improved, specific, and rapid detection of *Aspergillus* species in clinical specimens, we established a highly sensitive and *Aspergillus*-specific two-step PCR assay for peripheral blood and BAL samples (7, 8, 47). Some other groups have also previously described methodologically different assays with similar rates of sensitivity and specificity; comparative trials, however, are still lacking (17, 18, 23–25, 28, 31, 41, 42, 49, 53).

Besides fungal detection, quantification of the fungal burden is also of great clinical relevance, since the individual fungal burden may allow therapeutic monitoring. Meanwhile, the real-time PCR technology is successfully used in this field (22, 29, 30, 32, 34, 39). The LightCycler technology combines the fast in vitro amplification of DNA with immediate fluorescence detection of the amplicon. This allows the real-time quantification of the amount of DNA. A proven method for the highly specific detection of the PCR products uses the fluorescence resonance energy transfer system with sequence-specific hybridization probes.

To achieve an improved, specific, sensitive, and rapid method for quantification of the *Aspergillus fumigatus* fungal load in clinical samples, we established a LightCycler PCR assay to test blood and BAL samples. An optimal pair of primers and hybridization probes whose sequences were derived from the sequence of the *A. fumigatus* mitochondrial cytochrome *b* gene was selected. The sensitivity and specificity of the assay were evaluated, and clinical samples from immunocompromised patients were analyzed. The results of the assay were compared with the results of our previously published sensitive and specific nested PCR assay (47).

MATERIALS AND METHODS

Strains and growth conditions. Fungal and bacterial test strains were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM), Braunschweig, Germany; the American Type Culture Collection (ATCC), Manassas, Va.; or the Institute for Medical Microbiology and Hygiene, Klinikum Mannheim, University of Heidelberg, Heidelberg, Germany.

Prior to DNA extraction, the fungal cultures were grown in Sabouraud agar for 72 h at 30°C.

DNA extraction. Extraction of DNA from fungal cultures was performed as described previously (47). Extraction of DNA from the bacterial cultures was performed by ultrasonication of the pelleted bacteria and subsequent phenol-chloroform extraction, as described by Sambrook et al. (44).

Primer and hybridization probes for LightCycler-based amplification of *Aspergillus* DNA. The sequences of the PCR primers and probes were selected from the sequences of fungal mitochondrial cytochrome *b* genes.

The alignment of the mitochondrial cytochrome *b* genes of four *Aspergillus* spp. and four *Candida* spp. revealed several regions of minor homology between different species, with this homology being the presupposition for the design of the primers and hybridization probes. The alignment of the DNA sequences was performed by using the Geneworks program (Intelligenetics, Inc.) with standard algorithms.

Eight different primers and three pairs of hybridization probes were tested in 16 theoretically wise combinations. The optimum pairs of primers and hybridization probes were chosen for all subsequent PCR assays. From the sequence of the mitochondrial cytochrome *b* gene of *A. fumigatus* (GenBank accession no. AB025434), the forward primer was 5′-AATGCACGATACTGTAGGATCT G-3′ (primer AfLC2s) and the reverse primer was 5′-TGCATTGGATTAGCC ATAACA-3′ (primer AfLC2as). The amplified fragment was 194 bp in length. The sequences of the hybridization probes were selected from the region between the sequences of both primers of a primer pair. One probe (5′-TAATC TATCATAATTACCAGAAATACCTAAAGGA-3′; probe Cyt3A) was labeled at the 5′ end with the LightCycler Red 640 fluorophore. The other probe (5′-AATCTTTAAATACAAAGTAAGGAGCGAAAG-3′; probe Cyt3B) was labeled at the 3′ end with fluorescein. The primers and hybridization probes were obtained from TIB MOLBIOL, Berlin, Germany.

LightCycler PCR assay and quantification of target DNA. The LightCycler PCR and detection system (Roche Applied Science, Mannheim, Germany) was used for amplification and quantification of *A. fumigatus* DNA. The LightCycler hot-start PCR was performed in glass capillaries with a LightCycler Fast Start DNA Master Hybridization Probes kit (Roche Applied Science) as described by the manufacturer. The PCR mixture contained 1× Fast Start reaction mixture, in which the Fast Start *Taq* DNA polymerase, reaction buffer, deoxynucleoside triphosphates, and 10 mM $MgCl_2$ are combined with 3.5 mM $MgCl_2$, 20 pmol of each primer, and 60 nmol of hybridization probes. Mixing of the reagents for the PCR was accomplished under laminar flow.

PCR was performed in a volume of 20 μl under the following conditions: initial denaturation for 8 min at 95°C, 45 cycles of 4 s at 95°C, annealing for 8 s at 58°C, and enzymatic chain extension for 20 s at 72°C. Each PCR assay included a negative control consisting of H_2O without any template DNA to monitor for possible contamination. Furthermore, aliquots of DNA from healthy control persons were prepared concurrently with the DNA from clinical samples and were analyzed in parallel with negative controls by the LightCycler PCR.

A serially diluted standard of genomic *A. fumigatus* DNA was used for quantification. The amount of DNA corresponding to the copy number of the mitochondrial cytochrome *b* gene in *A. fumigatus* in the range of 10^6 to 10^1 copies was calculated, with 10^1 copies, corresponding to 13.2 fg of genomic *A. fumigatus* DNA or to 1 to 5 CFU per ml of blood, being detected. The logarithmic linear phase was distinguished from the background by online monitoring. The concentrations of *Aspergillus* DNA in unknown samples were calculated by comparing the cytochrome *b* gene copy numbers of the logarithmic linear phase of the sample with the copy numbers of the standards.

Examination of sensitivity and specificity of the LightCycler PCR assay. To determine the sensitivity of the LightCycler PCR assay, a serially diluted standard of genomic *A. fumigatus* DNA was used. As few as 10^1 copies of the mitochondrial *A. fumigatus* cytochrome *b* gene could be detected. This corresponds to 13.2 fg of genomic DNA or 1 to 5 CFU per ml of blood. DNA from several fungal and bacterial strains (Table 1) was subjected to the LightCycler PCR (Table 1) to determine the specificity of the assay. The cross-reactivities of the primers and the hybridization probes with human DNA were excluded by testing of DNA from 10 healthy control persons by the LightCycler PCR assay.

Clinical samples. Blood samples were obtained by venipuncture under sterile conditions and placed in a sterile vessel containing potassium EDTA to a final concentration of 1.6 mg EDTA per ml of blood. The sample volume was 5 to 7 ml.

To obtain BAL samples, bronchoscopy was performed according to the guidelines of the Deutsche Gesellschaft fuer Pneumologie (1), and BAL samples were obtained in a sterile vessel without conservation media. The mean sample volume was 10 ml.

Clinical samples were obtained from patients with proven or probable invasive aspergillosis according to the 2002 Consensus Conference definitions of invasive fungal infections in patients with cancer and recipients of hematopoietic stem cell transplants (2): proven invasive infection is based on histopathologic or cytopathologic examination showing hyphae from a needle aspiration or biopsy specimen with evidence of associated tissue damage or a positive culture result for a sample obtained by sterile procedure from a normally sterile site and a clinically or radiologically abnormal site consistent with infection. Patients with a probable invasive infection with molds are characterized by at least one host factor criterion (e.g., neutropenia or persistent fever refractory to appropriate antibiotics), one microbiological criterion (e.g., positive culture result for *Aspergillus* from sputum or BAL samples), and one major clinical criterion (or two minor clinical criteria) (e.g., clinical signs of lower respiratory tract, sinonasal, central nervous system, or disseminated infection or specific new lung infiltrates on computed tomography imaging).

Only clinical samples with positive results by the previously described nested PCR assay were examined (47).

Preparation of DNA from clinical samples. Five volumes of red cell lysis buffer (RCLB; 10× RCLB is 1.55 M NH_4Cl, 0.1 M NH_4HCO_3, and 1 mM EDTA [pH 7.4]) were mixed with 3 to 5 ml of peripheral blood, and the mixture was incubated on ice for 10 min. After lysis of the erythrocytes, the sample was centrifuged at 300 × *g* for 10 min. The supernatant was discarded, the leukocytes were washed once with 1× phosphate-buffered saline (PBS; 10× PBS is 1.4 M NaCl, 50 mM KCl, 90 mM $Na_2PO_4 \cdot 2\ H_2O$, and 20 mM KH_2PO_4 [pH 7.4]) and recentrifuged.

BAL samples were transferred to 1.5-ml tubes and centrifuged at 300 × *g* for 5 min. The sedimented cell material was processed as follows: the leukocyte

TABLE 1. PCR results for fungal and bacterial strains

Organism (strain)	LightCycler PCR result[a]
Fungal strains	
Aspergillus fumigatus (DSM 819 CS)	+
Aspergillus flavus (DSM 1959)	−
Aspergillus terreus (DSM 1958)	−
Aspergillus niger (DSM 63263)	−
Aspergillus versicolor (DSM 1943)	−
Aspergillus clavatus (DSM 3410)	+
Emericella nidulans (DSM 820)	−
Penicillium chrysogenum (DSM 844)	−
Penicillium expansum (DSM 1282)	−
Penicillium fumiculosum (DSM 1944)	−
Paecilomyces variotii (DSM 1961)	−
Rhizopus oryzae (DSM 854)	−
Candida glabrata (DSM 70614)	−
Candida tropicalis (DSM 1346)	−
Candida parapsilosis (DSM 70126)	−
Candida albicans (DSM 1386)	−
Candida krusei (DSM 70079)	−
Fusarium proliferatum (DSM 848)	−
Bacterial strains	
Streptococcus mitis (DSM 20568)	−
Streptococcus pneumoniae (DSM 20566)	−
Staphylococcus aureus (DSM 799)	−
Staphylococcus epidermidis (DSM 709)	−
Pseudomonas aeruginosa (ATCC 27853)	−
Klebsiella pneumoniae (DSM 681)	−
Enterobacter cloacae (DSM 6234)	−
Serratia marcescens (DSM 1636)	−
Haemophilus influenzae (DSM 4690)	−
Proteus mirabilis (DSM 788)	−
Escherichia coli (DSM 787)	−
Enterococcus faecalis (DSM 2570)	−
Enterococcus faecium (DSM 2146)	−

[a] Results from at least four separate experiments are shown.

pellet was resuspended in 300 μl of 1× PBS and incubated with 100 to 125 U of lyticase (50,000 U; Sigma, Deisenhofen, Germany) for 30 min at 37°C to achieve degradation of fungal cells. Proteinase K (500 to 1,000 μg; Roche Molecular Biochemicals, Mannheim, Germany) and 0.5% sodium dodecyl sulfate (Sigma) were added, and the suspension was incubated at 55°C for 1 h. Residual cell material was lysed by additional treatment with 100 μl of 2× *Aspergillus* extraction buffer (400 mM Tris-Cl, 1 M NaCl, 20 mM EDTA, 2% sodium dodecyl sulfate) for 30 min at 65°C. DNA was prepared under laminar flow. Fungal and human DNA was purified by conventional phenol-chloroform extraction (44). The DNA was precipitated with 70% (vol/vol) isopropanol. The DNA pellet was washed once with 70% ethanol and air dried, and the DNA concentration was measured by spectrophotometry at 260 and 280 nm.

RESULTS

Selection of primers and hybridization probes. The alignment of the mitochondrial cytochrome *b* genes of *Aspergillus* species, *Candida* species, and humans revealed several regions with low levels of homology between the different species. These regions were used for the selection of primers and hybridization probes. The alignment was the prerequisite for establishing a PCR assay specific for *Aspergillus*. The mitochondrial cytochrome *b* genes of *A. fumigatus* (GenBank accession no. AB025434), *Aspergillus flavus* (GenBank accession no. AB000596), *Aspergillus terreus* (GenBank accession no. AB000603), and *Aspergillus niger* (GenBank accession no. AB000597) were aligned with the mitochondrial cytochrome *b* genes of *Candida albicans* (GenBank accession no. AB0044919), *Candida parapsilosis* (GenBank accession no. AB044929), *Candida glabrata* (GenBank accession no. AB044922), *Candida tropicalis* (GenBank accession no. AB044930), and humans (GenBank accession no. M28016). Eight primers and three pairs of hybridization probes, identified by the alignment, were selected for optimization of the PCR assay. The primer pair and the hybridization probes that produced PCR products with the highest sensitivities and species specificities were primers AfLC2s and AfLC2as, which amplified a fragment of 194 bp, and hybridization probes Cyt3A and Cyt3B (Fig. 1).

Specificity, sensitivity, and reproducibility of the LightCycler PCR. To test the specificity of the LightCycler PCR assay, DNA extracted from a wide range of fungal and bacterial pathogens was used as the template. Only DNA from *A. fumigatus* (DSM 819 CS) and *Aspergillus clavatus* (DSM 3410) was detectable by the LightCycler PCR assay. All PCRs with the other fungal and bacterial strains were negative (Table 1). To determine the sensitivity, serially diluted *A. fumigatus* genomic DNA was used as the standard. The amount of DNA corresponding to the copy number of the mitochondrial cytochrome *b* gene in *A. fumigatus* in the range of 10^6 to 10^1 copies was calculated. A total of 10^1 copies of the mitochondrial cytochrome *b* gene, corresponding to 13.2 fg of *A. fumigatus* genomic DNA or 1 to 5 CFU per ml of blood, could be detected (47). Quantification was performed by monitoring the time point at which the logarithmic linear phase was distinguished from the background. Serially diluted samples of *A. fumigatus* genomic DNA corresponding to 1.3 ng to 13.2 fg were used as external standards in each run (Fig. 2A). The logarithm of the concentration of template DNA was plotted against the cycle numbers of the logarithmic linear phase. Calculation of the concentrations of fungal DNA in clinical samples was performed by comparing the cycle numbers of the external standards with the cycle numbers of the logarithmic linear phase of the samples (Fig. 2B).

The results of at least three independent experiments for the dilution series of *A. fumigatus* DNA were used to calculate the standard deviation and to prove the reproducibility of the LightCycler PCR assay (Table 2). The assay was linear to up to 4,233,600 copies, corresponding to 5 ng of genomic *A. fumigatus* DNA and about 1,136,363 CFU.

LightCycler PCR-based quantification of *Aspergillus* DNA in clinical samples. On the basis of the positive results of the nested PCR assay described previously (47), we investigated 10 BAL samples from nine neutropenic patients with hematological malignancies and 12 blood samples from seven neutropenic patients with hematological malignancies. One blood sample and one BAL sample from each of two additional patients were examined. In all patients, IPA was proven or probable according to the 2002 Consensus Conference definitions of invasive fungal disease (2). BAL samples were obtained within 5 days after the diagnosis of lung infiltrates; blood samples were mostly taken within the second week of the onset of fever in neutropenic patients.

By use of the LightCycler PCR assay, 10 fg of genomic *A. fumigatus* DNA corresponding to 1 to 5 CFU could be detected. Quantification of the LightCycler PCR assay should allow positive samples to be regarded in more detail in order to calculate the response to antifungal treatment.

At least three independent PCR runs were performed in

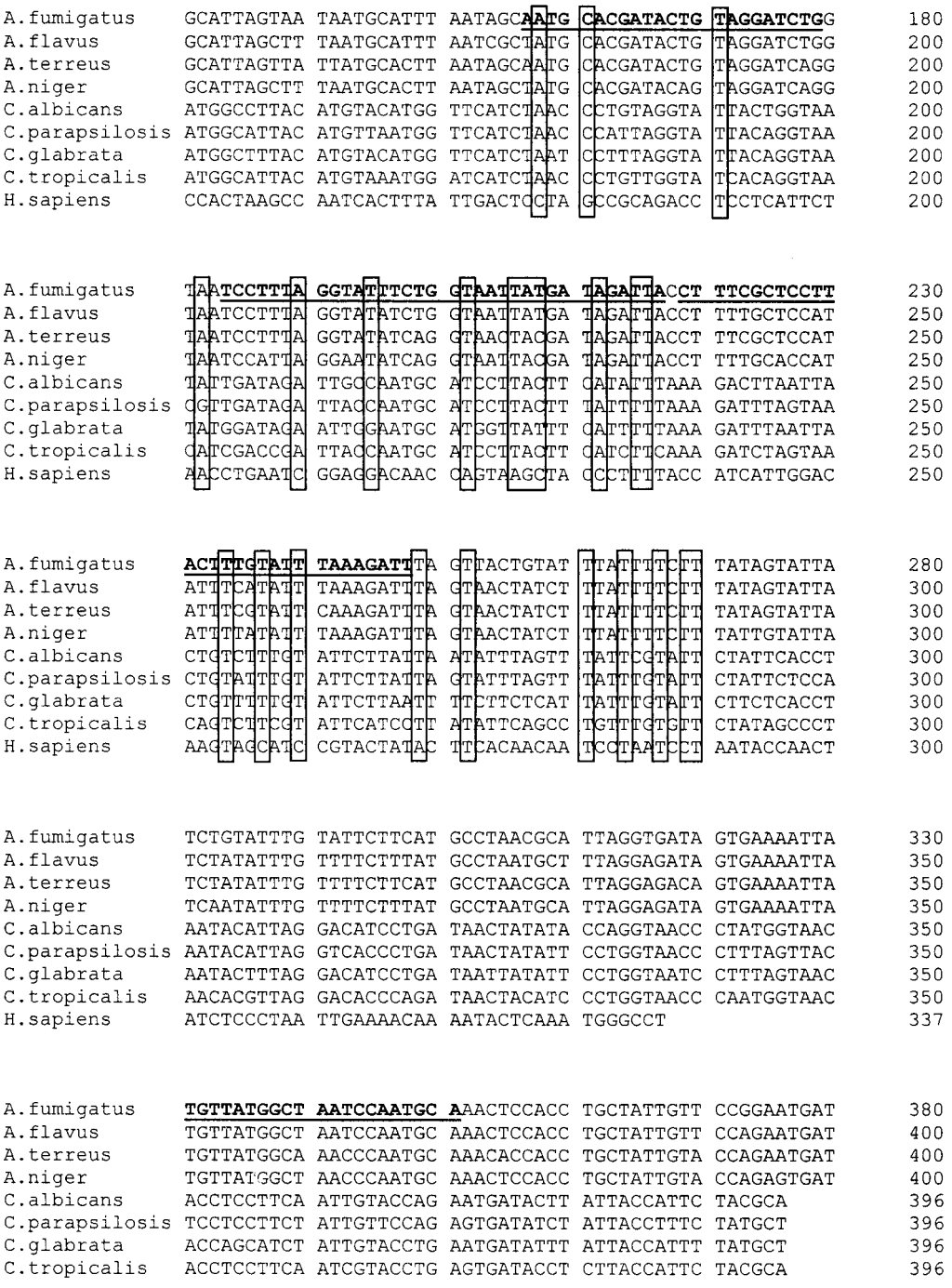

FIG. 1. Multiple-nucleotide-sequence alignment of mitochondrial cytochrome *b* genes of *A. fumigatus* (GenBank accession no. AB025434), *A. flavus* (GenBank accession no. AB000596), *A. terreus* (GenBank accession no. AB000603), *A. niger* (GenBank accession no. AB000597), *C. albicans* (GenBank accession no. AB0044919), *C. parapsilosis* (GenBank accession no. AB044929), *C. glabrata* (GenBank accession no. AB044922), *C. tropicalis* (GenBank accession no. AB044930), and humans (GenBank accession no. M28016). The locations of PCR primers and hybridization probes are underlined. Homologous regions are boxed.

these experiments. The results and patients characteristics are shown in Table 3. Altogether, 18 patients with positive results by the nested PCR assay were further characterized by the LightCycler PCR assay. For clinical evaluation, 12 BAL samples from 11 patients with malignant hematological diseases were investigated, mainly patients with acute leukemias (n = 8) with proven (n = 7) or probable invasive aspergillosis, according to the 2002 Consensus Conference definitions (2). Twelve of 12 BAL samples gave positive results by the LightCycler PCR assay, and the results were also validated by the nested PCR assay (47). The PCR-mediated quantification of the fungal burden showed 37 to 910,525 copies of the mito-

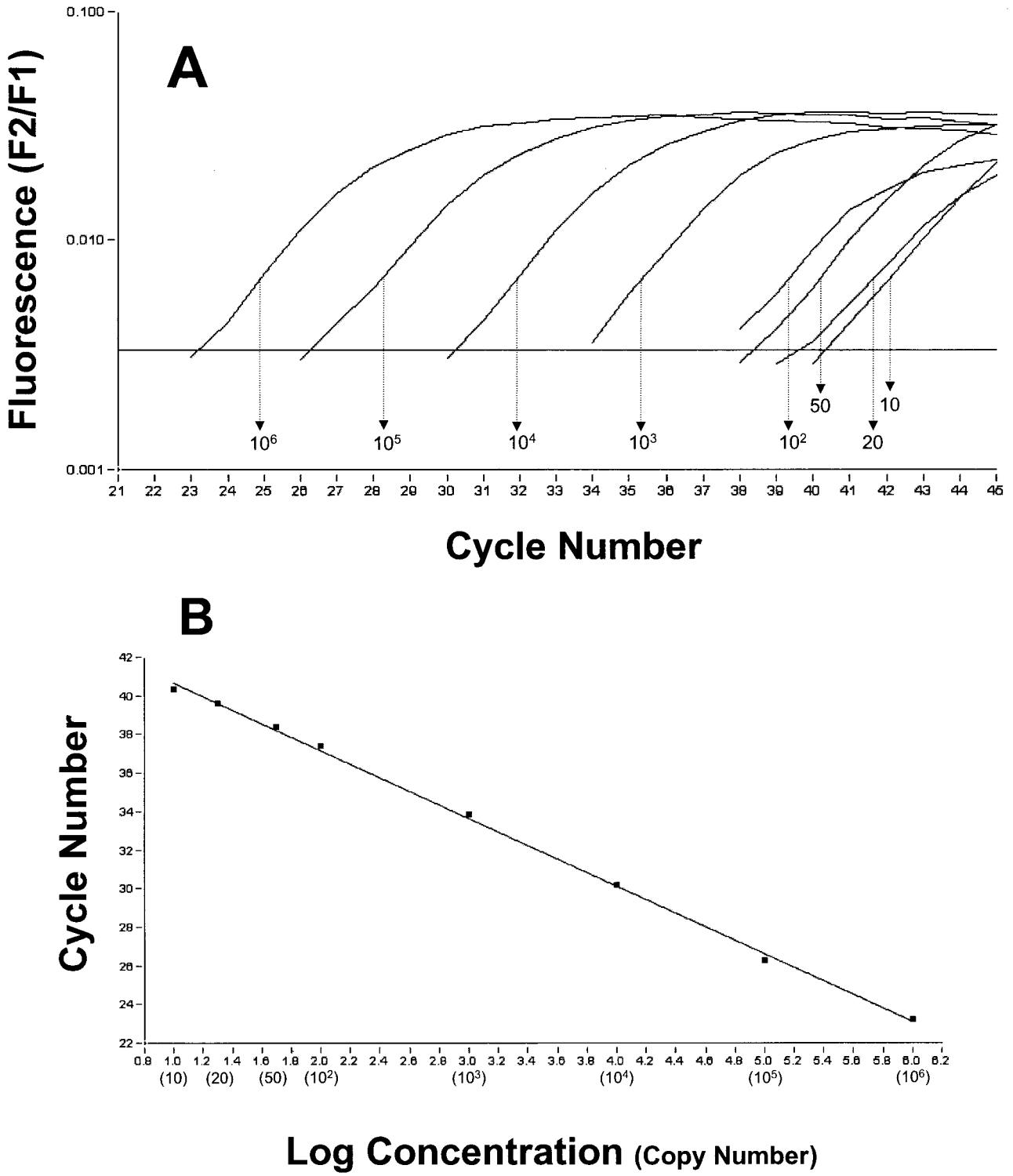

FIG. 2. (A) Quantification of a serially diluted standard of *A. fumigatus* DNA by the LightCycler PCR technique. (B) LightCycler PCR standard curve for serially diluted *A. fumigatus* DNA. The results of a representative evaluation are shown.

chondrial cytochrome *b* gene, corresponding to 15 to 269,018 CFU per ml of BAL sample. Twenty of 20 BAL samples from nonimmunocompromised patients were PCR negative.

Furthermore, we investigated 14 blood samples that were nested PCR positive from nine patients with malignant hematological diseases, mainly acute leukemias ($n = 7$) with proven ($n = 5$) or probable invasive aspergillosis, according to the 2002 Consensus definitions (2). Eight of these samples were LightCycler PCR negative. The sensitivity rate of the assay with these limited samples was 43%. The PCR-based quanti-

TABLE 2. Values determined for a serially diluted standard of *A. fumigatus* DNA

Defined standard copy no.	Standard copy no. determined by LightCycler PCR[a]	Standard deviation (%)
10^6	1,036,811 ± 121,925	11.7
10^5	98,751 ± 14,347	14.5
10^4	10,323 ± 1,076	10.4
10^3	1,008 ± 100	9.9
10^2	108 ± 17	15.7
50	49 ± 6	12.2
20	20.08 ± 2	10.0
10	11.70 ± 1.7	14.5

[a] Data represent means ± standard deviations from at least three separate experiments.

fication of the fungal burden showed 298 to 104,114 CFU per ml of blood. Fifty of 50 blood samples from healthy volunteers were PCR negative.

DISCUSSION

PCR has been shown to be a highly sensitive and specific diagnostic tool for the detection of *Aspergillus* species in clinical samples. To extend the diagnostic value of the previously described PCR assay (47) to the quantification of the pathogen load in order to improve treatment monitoring, we established a real-time PCR assay. This technology has successfully been applied for the diagnosis of infections that are hard to detect by culture assays (22, 29, 30, 32, 34, 39).

To establish the LightCycler PCR assay, we selected primers and hybridization probes whose sequences were derived from the sequence of the multicopy mitochondrial cytochrome *b* gene, for which the degree of homology between *Aspergillus* and *Candida* species is the lowest. The LightCycler PCR assay featured enhanced species specificity due to the use of a specific primer pair and two independent hybridization probes. No cross-amplification was observed with DNA from humans, *Candida* or *Penicillium* species, or a wide range of other fungal or bacterial pathogens (Table 1).

By use of this assay in dilution series in vitro, 10 copies of the mitochondrial cytochrome *b* gene corresponding to 13.2 fg of genomic *A. fumigatus* DNA or 1 to 5 CFU could be detected and quantified, whereas the detection threshold of the previously described nested PCR assay (47) is 10 fg. The reproducible detectable 50-copy standard is used as minimal threshold for quantification, as the 10-copy standard was not constantly measurable in the linear range of the PCR assay. Fifty copies corresponds to 66 fg of *A. fumigatus* genomic DNA, which is approximately 15 CFU.

To assess the clinical implications and applicability of this assay, we examined clinical samples from 18 patients with malignant hematological diseases, including 12 BAL samples from 11 patients and 14 blood samples from 9 patients. Both BAL and blood samples that tested positive in our previously described nested PCR assay were obtained from patients with proven and probable IPA and had a wide range of pathogen burdens. As bronchoscopy was performed early in the clinical course of the infection, the fungal load in BAL specimens was not correlated to the outcome. Eight blood samples positive by

TABLE 3. PCR results for patient blood and BAL samples

Patient no.	Sample	Time sample was taken (day after date of diagnosis)	Diagnosis[a]	Diagnostic significance[b]	LightCycler PCR gene copy no./ml[c]	Corresponding no. of CFU/ml[c]	Nested PCR result	Outcome
1	1 (BAL)	+2	AML	Proven	37 ± 9	15 ± 4	+	Death, AML
1	2 (blood)	+3	AML	Proven			+	Death, AML
2	BAL	+3	Hodgkin's disease	Proven	17,837 ± 1,605	5,270 ± 474	+	Death, HD[d] relapse
3	BAL	+3	AML	Proven	13,670 ± 2,324	4,038 ± 686	+	Death, AML
4	BAL	+1	ALL	Proven	910,525 ± 81,947	269,018 ± 24,211	+	Death, IPA
5	BAL	+4	AML	Proven	597 ± 280	176 ± 83	+	Death/bacteremia
6	BAL	+4	AML	Probable	385 ± 254	114 ± 75	+	Death, IPA
7	1 (BAL)	+4	CML blast crisis	Proven	922 ± 396	272 ± 117	+	Death, IPA
7	2 (BAL)	+13	CML blast crisis	Proven	2,697 ± 863	797 ± 255	+	Death, IPA
8	BAL	+3	AML	Probable	920 ± 223	271 ± 66	+	Death, IPA
9	BAL	+1	AML	Probable	44,687 ± 6,740	13,202 ± 1,991	+	Survival
10	1 (BAL)	+2	Chronic neutropenia	Proven	55,171 ± 26,157	16,300 ± 7,727	+	Death, IPA
10	2 (blood)	+2	Chronic neutropenia	Proven	1,010 ± 707	298 ± 208	+	Death, IPA
11	BAL	+4	AML	Probable	2,704 ± 243	799 ± 72	+	Survival
12	blood	+8	ALL	Probable	11,790 ± 6,930	3,483 ± 2,047	+	Survival
13	1 (blood)	+5	CLL	Proven			+	Death, IPA
13	2 (blood)	+10	CLL	Proven	28,491 ± 8,262	8,418 ± 2,441	+	Death, IPA
14	1 (blood)	+9	ALL relapse	Proven			+	Death, ALL
14	2 (blood)	+11	ALL relapse	Proven			+	Death, ALL
14	3 (blood)	+15	ALL relapse	Proven			+	Death, ALL
15	blood	+8	AML	Probable			+	Death, AML
16	1 (blood)	+9	AML relapse	Proven			+	Death, AML
16	2 (blood)	+13	AML relapse	Proven	78,000 ± 3,013	23,045 ± 890	+	Death, AML
17	blood	+5	AML	Probable			+	Survival
18	1 (blood)	+8	AML	Probable	106,360 ± 27,654	31,424 ± 8,170	+	Death, AML
18	2 (blood)	+10	AML	Probable	352,388 ± 96,671	104,114 ± 28,561	+	Death, AML

[a] AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; CML, chronic myeloid leukemia; CLL, chronic lymphoblastic leukemia.
[b] As described elsewhere (2).
[c] Data represent means ± standard deviations from three separate experiments. Where no value is given, the result was negative.
[d] HD, Hodgkin's disease.

the nested PCR assay were LightCycler PCR negative. This could be due to the exclusive specificity of the assay for *A. fumigatus* and *A. clavatus*, in contrast to our nested PCR assay, which detects a wider range of *Aspergillus* species, or the facts that the fungal loads of these samples were in the range between the thresholds of the nested and the LightCycler PCR assays, i.e., between 5 and 15 CFU. Any in vitro attempts to improve the sensitivity rates led to an impairment of the species specificity of the LightCycler PCR assay. As the sensitivity of the LightCycler PCR assay was inferior to that of the nested PCR assay (47), the LightCycler PCR assay is less appropriate as a diagnostic tool early in the course of infection but allows quantification of the fungal burden in clinical samples. To compensate for the lack of an overlap of the detection thresholds of the two PCR methods, we actually screened patient samples by our nested PCR assay, which provided the highest sensitivity and general specificity for the detection of *Aspergillus* species. Samples that tested positive by the nested PCR were subsequently quantified by the LightCycler PCR assay.

The LightCycler PCR assay was also developed to monitor antifungal treatment. Monitoring of the clinical course of a patient with acute myeloid leukemia (patient 18), who developed specific lung infiltrates during induction therapy (probable infection), showed that the first blood sample was negative by the two PCR assays but that the second and third samples were positive by both PCR assays. At this time the patient developed fever and lung infiltrates and was treated with antibacterial antibiotics and amphotericin B intravenously. The fungal load in the first positive blood sample was 31,424 CFU/ml of blood; that in the second sample was 104,114 CFU/ml of blood. A high-resolution computed tomography scan showed specific lung infiltrates indicating IPA at this time. PCR results became negative during antifungal treatment and recovery of neutrophils (Table 3). These preliminary data may point to the clinical validity of the assay in this field.

Until now, descriptions of two clinically based real-time PCR assays that used the 18S rRNA gene of *Aspergillus* species and two different detection systems have been published (29, 34). For determination of the species specificities of the two assays, only DNA from *Candida* species and humans was tested for cross-reactivity, whereas in our novel PCR assay, cross-amplification with a wide range of bacterial and fungal pathogens could be definitively excluded. The rates of sensitivity of our assay and the previously published assays are comparable. For clinical evaluation, only blood samples were tested by both previously published assays.

To monitor antifungal treatment, the screening of blood samples is clinically more applicable, because the samples can be obtained repeatedly and by noninvasive means. Moreover, the testing of BAL samples has been shown to be a valuable diagnostic tool to confirm IPA early, and tests with BAL samples have both with higher sensitivities and higher specificities than tests with blood samples (7), because *Aspergillus* infections are primarily airborne pulmonary infections and only secondarily spread to the blood. We therefore first examined BAL samples from selected patients with probable and proven IPA by the novel LightCycler PCR assay and confirmed the positivity of the nested PCR assay results for 12 of 12 samples, whereas with blood samples the diagnostic value of the LightCycler PCR assay for the detection of infection was definitely lower, so we examined blood samples first by the nested PCR assay. A trial being conducted focuses on this strategy and on the correlation between the fungal loads in blood and BAL samples and computed tomography findings for the lung. A clinical evaluation investigating the suitability of quantitative PCR results for blood samples for monitoring of therapy is ongoing as well. In summary, a highly specific and sensitive LightCycler-based real-time PCR assay with blood and BAL samples from high-risk patients was established for the rapid and early detection of *Aspergillus* species and the quantification of the fungal load in order to monitor antifungal treatment.

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