

Use of Real-Time PCR on Blood Samples for Diagnosis of Invasive Aspergillosis

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We developed a new quantitative system for diagnosis of invasive pulmonary aspergillosis (IPA) using real-time automated polymerase chain reaction (PCR). Intra-assay and interassay precision rates for *in vitro* examination were 2.53% and 2.20%, respectively, and the linearity of this assay was obtained when there were >20 copies/well. We examined 323 samples taken from 122 patients with hematological malignancies, including 33 patients with IPA and 89 control patients. Blood samples were subjected to PCR antigen detection methods, using enzyme-linked immunosorbent assay (ELISA) and determination of plasma (1→3)- β -D-glucan (BDG) concentration. The sensitivities of PCR, ELISA, and BDG measurement for diagnosis of IPA were 79%, 58%, and 67%, respectively; the specificities were 92%, 97%, and 84%. Positive findings on PCR preceded those of computed tomography by -0.3 ± 6.6 days, those of BDG measurement by 6.5 ± 4.9 days, and those of ELISA by 2.8 ± 4.1 days. Real-time PCR was sensitive for IPA diagnosis, and quantitation was accurate.

Invasive pulmonary aspergillosis (IPA) is a fatal complication in immunocompromised hosts who undergo intensive cytotoxic chemotherapies. The survival of these patients depends on early diagnosis and prompt initiation of therapeutic measures [1], but mycological and histopathological diagnosis is rarely established, because blood culture is not sufficiently sensitive for this disease [2], and invasive procedures are necessary to obtain pathological evidence. The difficulty of making an early diagnosis of IPA is a critical problem. Efforts have been made, therefore, to develop reliable noninvasive diagnostic techniques.

Use of CT of the chest and antigen-detection methods have been advocated for early diagnosis of IPA. The most characteristic findings of CT in IPA are a halo of ground-glass attenuation around focal nodules and air-crescent formation [3]. Both signs are relatively specific to IPA in febrile neutropenic patients, but they have also been reported in association with a variety of other diseases [4]. Thus, it is impossible to establish a definite diagnosis of IPA on the basis of the findings of CT scans of the chest. Detection of circulating *Aspergillus* or fungal antigens is another useful method for the diagnosis of IPA. Currently, 3 methods are used: the latex agglutination test, ELISA, and measurement of the plasma (1→3)- β -D-glucan (BDG) concentration. The latex agglutination test and ELISA are commercially available for use in detecting circulating galactomannan antigen. BDG is a ubiquitous component of fungi [5], and the determination of its plasma concentration is another useful screening method for deep mycosis, including IPA [6]. The BDG assay is widely used in Japan. Because these blood tests detect circulating fungal components, their specificities are as high as 90% [6–8]. However, these tests yield positive results only at ad-

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Informed consent was obtained from all patients included in this study. We followed the guidelines for clinical research of the Graduate School of Medicine, University of Tokyo; Toranomon Hospital; and Tokyo Metropolitan Komagome Hospital.

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vanced stages of infection in a majority of patients with IPA, which is a critical problem [9, 10].

PCR may be useful for the detection of fungal nucleic acids because it can be more sensitive than the current antigen detection methods. Numerous protocols have been established for the diagnosis of *Aspergillus* infection by use of PCR [11–13], and they have been successfully used to detect *Aspergillus* DNA in bronchoalveolar lavage fluid [14] and in serum obtained from patients with IPA [12]. Although the conventional PCR assay is highly sensitive, with a detection limit of 10 fg of *Aspergillus*-specific DNA [12], it does not provide quantitative information on the fungal burden. Such information is a requisite for the management of IPA. Real-time PCR is a quantitative assay reported by Heid et al. in 1996 [15]. This method measures PCR product accumulation through a dual-labeled fluorogenic probe (TaqMan Probe) and provides accurate and reproducible quantitation of gene copies [15]. We recently developed a new quantitative diagnostic system using this exonuclease-based PCR assay as a rapid alternative to conventional PCR for the diagnosis of IPA. This is the first report on the use of quantitative real-time PCR for the diagnosis of IPA.

MATERIALS AND METHODS

Extraction of DNA from blood samples. Fungal DNA was extracted from 200 μL of whole blood or plasma in a biosafety hood in a separate room provided with equipment exclusively used for DNA extraction [16]. The procedures used for DNA extraction have been reported elsewhere [17]. To avoid contamination, amplicons were never processed in this area.

Blood samples were stored at -80°C and thawed to 4°C before testing. Freeze/thaw cycles were not repeated, and we used newly thawed samples in this study. Our preliminary study showed that sensitivity of PCR increased as the blood volumes increased (data not shown) and, therefore, that it is preferable to use >5 mL of blood for PCR samples. However, we used 200 μL of blood or plasma for PCR samples, because the volume of most stored samples was 200–500 μL .

Amplification and detection of *Aspergillus* DNA. Sequences of PCR primers and probes were selected on the basis of the sequences of fungal 18S rRNA genes in the GenBank database. The primers and probes hybridized a consensus sequence for *Aspergillus* species. The forward primer was 5'-TTG-TGGAGTGATTGTCTGCT-3', and the reverse primer was 5'-TCTAAGGGCATCACAGACCTG-3'. The TaqMan probe selected the region between the primers, which was fluorescence-labeled with FAM (6-carboxy-fluorescein) at the 5' end as the reporter dye and TAMRA (6-carboxy-teremethyl-rhodamine) at the 3' end as the quencher (FAM-TCGGCCCTTAAATAGC-CCGGTCCGC-TAMRA). The primers and the TaqMan probe were obtained from FASMAC.

Procedures for PCR and detection of the PCR product have been reported elsewhere [17]. DNA from an *Aspergillus fumigatus* isolate was amplified with both primers and inserted into pCR2.1 TA Cloning vector (Invitrogen). We used this plasmid as the standard in this study.

Quantitation of *Aspergillus* DNA was performed with a serially diluted standard in the range of 1×10^1 – 1×10^8 copies/well, and the copy numbers of *Aspergillus* species were calculated by Sequence Detection System v. 1.6.3. software (PE Biosystems). Fluorescence intensity was measured in each well every 7 s, and the result was deemed to be positive when the fluorescence intensity exceeded 10 times the baseline fluorescence (figure 1). The threshold cycle value was defined as the PCR cycle number at that point.

In vitro examination of sensitivity and specificity of real-time automated PCR. To determine the sensitivity of real-time automated PCR for detection of *Aspergillus*, 100 copies/ μL of standard DNA were serially diluted with TE buffer (10 mM Tris-HCl [pH 8.0] and 1 mM EDTA). The diluted DNA samples were measured 5 times.

To determine the specificity, DNA from *A. fumigatus*, *Aspergillus niger*, *Aspergillus terreus*, *Candida albicans*, *Candida tropicalis*, *Candida krusei*, *Candida parapsilosis*, *Candida glabrata*, and *Candida guilliermondii* isolates was subjected to real-time PCR.

In vivo comparison of real-time PCR with ELISA and BDG measurement. To compare the sensitivity and specificity of real-time PCR with those of other blood tests for the diagnosis of IPA, we investigated blood samples obtained from patients

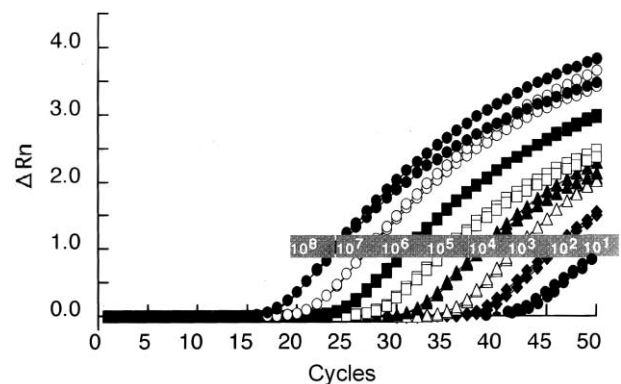


Figure 1. Amplification profile of standards for *Aspergillus* real-time PCR; 10^1 – 10^8 copies/well (black bar) of standard *Aspergillus* DNA were amplified in duplicate for 50 cycles. The normalized reporter (Rn) value was calculated by dividing the emission intensity of the reporter dye by the emission intensity of the reference dye included in the TaqMan Universal PCR Master Mix, which does not participate in the exonuclease-based PCR assay. ΔRn is the difference between the $\text{Rn}+$ value and the $\text{Rn}-$ value, where $\text{Rn}+$ is the Rn value of a reaction that contains all components, including the template, and $\text{Rn}-$ is the Rn value obtained from early cycles, before a detectable increase in fluorescence, during real-time PCR.

in our hematology wards who underwent chemotherapy as treatment for hematological malignancies or severe aplastic anemia.

All the blood samples were subjected to real-time PCR, ELISA (Platelia *Aspergillus*; Diagnostic Pasteur), and BDG determination (Fungi-Tec; Seikagaku Corporation). These tests were performed as described elsewhere [5, 18]. When an optical density ratio of ELISA >1.5 was observed in at least 2 samples, we classified the ELISA results for those patients as positive. The cutoff level for the BDG measurement was 20 pg/mL [6]. When these tests were performed more than once, the highest value in each febrile episode was used in the analysis of data.

Conditions of hospitalization. All patients who underwent bone marrow transplantation (BMT) and some patients who were receiving high-dose chemotherapy were cared for in protective isolation in rooms equipped with high-efficiency particulate air filters throughout the period of neutropenia. All the patients received either fluconazole (200 or 400 mg/day) or itraconazole (200 mg/day) as antifungal prophylaxis. We managed neutropenic fever according to procedures described in the report by Pizzo [19].

Blood sampling, chest CT scanning, and fungal cultures. From August 1996 through March 1999, blood samples were stored when we suspected, on the basis of clinical and radiological findings, that patient had IPA. From April 1999 through April 2000, we prospectively examined patients who had antibiotic-resistant fever. Blood samples were drawn weekly and subjected to ELISA, BDG assay, and PCR. Cultures were performed on samples from the throat, urine, feces, blood, and sputum when antibiotic-resistant fever developed. We performed chest CT scans when patients had any signs of pulmonary infection or of antibiotic-resistant fever. Patients with conditions that required chest CT have been reported elsewhere [20]. Bronchoalveolar lavage is not usually performed in our institutions if patients have either thrombocytopenia or neutropenia.

Diagnostic criteria for IPA. We made a diagnosis of definite IPA when patients had histological evidence of tissue invasion by branched septate hyphae, when a patient's condition did not respond to antibacterial agents, and when testing of sputum samples or biopsy specimens or autopsy results were positive for *Aspergillus* species. We classified patients who developed fever but showed no signs of IPA as control patients. Patients who were suspected of having IPA on the basis of radiological findings [3] but who had no histological evidence of IPA were excluded from this study.

Statistical analyses. Data were analyzed for statistical significance by the 2-tailed Fisher exact test or by the χ^2 test. Correlation analysis was performed using Kendall's nonparametric correlation coefficient. The level of statistical significance was set at $P < .05$.

RESULTS

In vitro sensitivity, reproducibility, and specificity of real-time PCR. Copy numbers of *Aspergillus* DNA and threshold cycle values of the serially diluted standard were plotted on the X-axis and the Y-axis, respectively, of the graph shown in figure 2. The correlation coefficient was .988. The intra-assay coefficient of variance (CV) was <5% (0.83%–4.7%) between 20 and 10^{10} copies/well and 12.8% at 10 copies/well. Although we could detect as few as 10 copies/well, the sensitivity of real-time PCR as a quantitative assay was reduced to 20 copies/well. When we measured samples that contained 10^2 , 10^5 , and 10^6 copies of *Aspergillus* DNA 5 times, interassay CV values (\pm SD) were $2.20\% \pm 0.77\%$, $1.33\% \pm 0.36\%$, and $2.01\% \pm 0.40\%$, respectively. DNA obtained from *A. fumigatus*, *A. niger*, *A. terreus*, *Aspergillus flavus*, and *Aspergillus oryzae* were successfully amplified, but none of the *Candida* species were detected.

Patient characteristics. We examined 122 patients who underwent chemotherapy for treatment of hematological malignancies or severe aplastic anemia, of whom 48 were investigated prospectively. A total of 33 patients had definite IPA, and the other 89 patients served as control patients. Patient characteristics are shown in table 1. The organs infected by *Aspergillus* were lung parenchyma ($n = 31$), tracheal trees ($n = 3$), CNS ($n = 10$), kidney ($n = 6$), liver ($n = 7$), spleen ($n = 5$), gastrointestinal tract ($n = 5$), thyroid gland ($n = 2$), and adrenal gland ($n = 2$). *Aspergillus* involvement was limited to the lung in 16 patients, and systemic dissemination was observed in the other 17 patients. Complications in the control group were septicemia due to *C. tropicalis* ($n = 2$), septicemia due to *C. glabrata* ($n = 2$), pneumonia due to *C. albicans* ($n = 1$), pneumonia due to *C. tropicalis* ($n = 2$), cytomegalovirus pneumonia ($n = 2$), cytomegalovirus viremia ($n = 2$), respiratory syncytial virus pneumonia ($n = 1$), *Pneumocystis carinii* pneumonia ($n = 5$), bacterial septicemia ($n = 15$), bacterial pneumonia ($n = 25$), bacterial enterocolitis ($n = 7$),

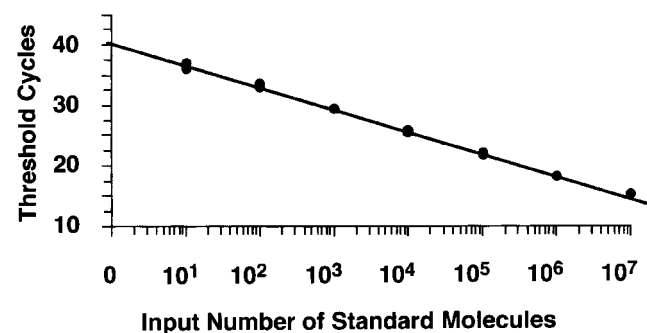


Figure 2. Standard curve for *Aspergillus* real-time PCR. Threshold cycle values were plotted against various numbers of standard DNA copies. The slope was -3.54 cycles/log decade. The correlation coefficient was .988.

Table 1. Characteristics of patients with definite invasive pulmonary aspergillosis (IPA) and control patients.

Characteristic	Patients with definite IPA (n = 33)	Control patients (n = 89)	P
Age, years (range)	50.5 (22–80)	47.6 (17–80)	.6761
Sex, male/female	26/7	67/22	.8123
Primary disease			.9667
Acute leukemia ^a	21	57	
Other	12	32	
Status of primary disease			.0251 ^b
Remission	10	48	
Refractory	23	41	
Protective isolation			.9667
Yes	12	32	
No	21	57	
Duration of neutropenia, days	38.9 (3–82)	17.8 (3–55)	<.0001 ^b
Steroid use			.7759
Yes	5	12	
No	28	77	
Status of acute graft-versus-host disease			.6773
Grade 0–I	7	22	
Grade II–IV	3	13	
Use of antifungal agents ^c			<.0001 ^b
Yes	31	42	
No	2	47	
Treatment for hematological malignancy			.1529
Bone marrow transplantation	12 ^d	47	
Conventional chemotherapy	21	42	
Type of transplant			.7119
Allogeneic	10	35	
Autologous	2	12	
Type of invasive aspergillosis ^e			
Disseminated	21		
Localized	12		
Outcome			<.0001 ^b
Death	25	30	
Survival	8	59	

NOTE. Data are no. of patients unless indicated otherwise.

^a Acute leukemia includes acute myeloblastic leukemia, acute lymphoblastic leukemia, adult T cell leukemia, and chronic myelocytic leukemia at blastic crisis or accelerated phase.

^b Statistically significant.

^c Antifungal agents included itraconazole and amphotericin B as prophylactic, empiric, or therapeutic administration. Antifungal agents that lack anti-*Aspergillus* activity, such as fluconazole, were not included in this analysis.

^d IPA occurred during neutropenia in 3 patients, during graft-versus-host disease in 6 patients, and after relapse in 3 patients.

^e Cases of definite IPA were divided into 2 groups: disseminated IPA and localized IPA. Infection by *Aspergillus* species, with multiple, noncontiguous organ involvement, was classified as disseminated IPA. IPA localized to a single organ, with or without contiguous invasion to the adjacent organs, was classified as localized IPA.

Table 2. Results of chest CT scan, real-time PCR, ELISA, and plasma (1→3)-β-d-glucan (BDG) assay for patients with invasive pulmonary aspergillosis (IPA).

Patient no.	Age, years	Sex	Primary disease	Disease status	Outcome	CT scan		PCR		BDG assay		ELISA	
						Findings ^a	Interval, days ^b	Maximum levels, copies/mL	Interval, days ^b	Maximum levels, pg/mL	Interval, days ^b	Maximum levels, OD ratio	Interval, days ^b
1	66	M	AML	Refractory	Death	—	7	20,000 ^c	7	213.7	20	5.6	20
2	47	M	CLL	Refractory	Death	Halo	12	20,000 ^c	6	243.4	6	5.1	6
3	45	M	ALL (auto-PBSCT)	Refractory	Death	—	16	13,500	22	4.6	NA	5.0	22
4	58	M	AML	Refractory	Death	Halo	12	11,500 ^c	22	76.4	29	5.0	29
5	69	F	CML	Refractory	Death	Halo	31	10,700	34	113.0	30	5.0	34
6	37	F	AML (allogeneic BMT)	Refractory	Death	Halo, air-crescent sign	9	6250	37	68.4	49	2.2	43
7	66	M	NHL	CR	Death	Halo, air-crescent sign	5	4500	12	690.0	19	4.4	12
8	56	F	MM	Refractory	Death		14	4300	14	19.9	NA	1.7	NA
9	44	M	CML (allogeneic BMT)	CR	Death	Thickening of the tracheobronchial wall	15	3350 ^c	3	1287.0	12	2.9	6
10	64	M	ALL	Refractory	Death	—	9	3300	16	90.9	16	0.3	NA
11	46	F	AML	Refractory	Death	Halo	3	2600	8	34.3	8	2.9	8
12	23	M	ALL (allogeneic BMT)	Refractory	Death	—	11	2000 ^c	11	27.0	18	1.9	18
13	24	M	SAA (allogeneic BMT)	CR	Survival	Halo, air-crescent sign	8	920	16	13.8	NA	0.8	NA
14	44	F	MDS (allogeneic BMT)	CR	Survival	Halo	10	800 ^c	10	146.6	10	2.2	10
15	50	M	MDS (allogeneic BMT)	Refractory	Death	Halo	12	720	18	510.6	24	2.8	24
16	80	M	NHL	Refractory	Death	Halo	3	600	7	33.8	7	4.9	7
17	50	M	CML	Refractory	Death	—	3	580	10	0.0	NA	0.2	NA

18	49	M	ALL	Refractory	Death	Halo	7	400	12	3	NA	0.1	NA
19	63	M	AML	Refractory	Death	Wedge-shaped consolidation	29	320	9	35.5	NA	4.5	9
20	69	M	ALL	Refractory	Death	—	22	200	22	52.6	22	2.1	22
21	45	M	CML (allogeneic BMT)	Refractory	Survival	Halo	12	100 ^c	12	18.9	NA	1.2	NA
22	50	M	NHL	Refractory	Death	—	8	100 ^c	8	26.8	12	0.5	NA
23	70	M	AML	Refractory	Death	—	12	100 ^c	20	95.3	32	0.4	NA
24	23	M	ALL (allogeneic BMT)	CR	Survival	Air-crescent sign	17	60 ^c	12	1.3	NA	0.1	NA
25	47	M	AML	CR	Survival	—	4	50 ^c	12	4.5	NA	0.5	NA
26	38	M	NHL (autologous PBSCT)	CR	Survival	Air-crescent sign	18	40	32	3.1	53	0.6	NA
27	46	M	ALL	Refractory	Death	Halo	12	0	NA	3.0	NA	3.9	12
28	34	F	NHL	CR	Death	—	21	0	NA	63.3	21	3.8	21
29	62	M	AML	Refractory	Survival	—	12	0 ^c	NA	20.9	12	2.2	12
30	76	M	AML	Refractory	Death	—	16	0 ^c	NA	449.7	12	2.1	12
31	27	M	MDS (allogeneic BMT)	CR	Survival	Air-crescent sign	13	0 ^c	NA	25.2	8	1.1	NA
32	76	M	AML	Refractory	Death	—	7	0 ^c	NA	13.8	NA	0.5	NA
33	22	F	MDS (allogeneic BMT)	CR	Death	—	4	0	NA	26.4	16	0.1	NA

NOTE. ALL, acute lymphoblastic leukemia; AML, acute myeloblastic leukemia; BMT, bone marrow transplantation; CLL, chronic lymphocytic leukemia; CML, chronic myelogenous leukemia; CR, complete remission; MDS, myelodysplastic syndrome; NA, not applicable; NHL, non-Hodgkin's lymphoma; OD, optical density; PBSCT, peripheral blood stem cell transplantation; SAA, severe aplastic anemia.

^a Multiple or single nodular consolidation was observed in all the patients with IPA.

^b Time interval between the onset of fever and the day on which a positive test result was obtained. For some patients, no positive test result was obtained (NA).

^c Samples were prospectively collected once per week after patients developed antibiotic-resistant fever. From August 1996 through March 1999, blood samples were stored when we suspected IPA on the basis of clinical and radiological findings. From April 1999 through April 2000, we prospectively examined patients who had antibiotic-resistant fever.

graft-versus-host disease ($n = 7$), progression of hematological malignancy ($n = 13$), and fever of unknown origin ($n = 6$).

Results of real-time PCR, ELISA, and BDG measurement.

We obtained 102 blood samples, including 92 whole blood and 10 plasma samples, from the 33 patients with IPA, and 221 samples, including 198 whole blood and 23 plasma samples, from the 89 control patients. We did not divide blood samples into whole blood and plasma to investigate which of the 2 samples was more suitable for use in PCR for detection of fungal DNA. On average, 3.1 (range, 1–8) and 2.5 (range, 1–7) samples were drawn from patients in the IPA group and in the control group, respectively. Table 2 shows the results of these tests in each patient with IPA.

Of the 33 patients with IPA, 26, 19, and 22 had positive results of PCR, ELISA, and BDG measurement, respectively, and 7, 3, and 14 of the 89 control patients had positive results of these assays. The sensitivities of real-time PCR, ELISA, and BDG measurement were 79%, 58%, and 67%, respectively. The specificities of the assays were 92%, 97%, and 84%. The positive predictive values were 79%, 86%, and 61%, respectively, and the negative predictive values were 92%, 86%, and 87%.

Of the 102 samples obtained from the 33 patients with IPA, 34 were drawn before empirical treatment with amphotericin B was started or in patients who were not receiving itraconazole prophylaxis. Fungal DNA was found, using PCR, in 12 of the 34 samples. Sixty-eight samples were collected after initiation of empirical amphotericin B treatment or during prophylactic administration of itraconazole. Fungal DNA was found, using PCR, in 20 of those 68 samples. Neither amphotericin B nor itraconazole administration seemed to influence the PCR results ($P = .6516$).

Results of fungal culture and CT scanning. Chest CT scans were performed on all 33 patients with IPA. All of the patients showed some abnormal findings in CT scans. The most common CT sign was multiple nodular consolidations. Either halo or air-crescent signs were observed in 12 patients with IPA. No control patients showed these signs.

All of the patients with IPA had microbiological evidence of *Aspergillus* infection. The organism was isolated from culture of samples obtained before death from 13 patients. Twelve cultures were of sputum samples, and 1 was of a biopsy specimen from the lung. *Aspergillus* infection was identified after the patient's death in the remaining 20 patients. The causative *Aspergillus* species were *A. fumigatus* ($n = 20$), *A. flavus* ($n = 4$), *A. niger* ($n = 2$), and *Aspergillus* species that were not subclassified ($n = 6$).

We show the association between PCR and other examinations in table 3. A significant association between PCR and the other examinations was not observed.

Interval between onset of fever and day on which each test yielded positive results. After retrospective study of 18 pa-

Table 3. Association between the results of PCR and other examinations for aspergillosis.

Test, result ^a	Results of PCR, no. of patients		P
	Positive	Negative	
CT scan			
Positive	9	3	
Negative	17	4	.6856
Antemortem fungal culture			
Positive	11	2	
Negative	15	5	.6756
ELISA			
Positive	15	4	
Negative	11	3	.9792
Plasma (1→3)-β-D-glucan assay			
Positive	17	5	
Negative	9	2	.7633

^a "Positive" denotes patients who showed positive results more than once throughout the clinical course; "negative" denotes patients for whom results were negative throughout the clinical course.

tients, we prospectively evaluated 15 patients who had IPA. For these patients, CT scans, PCR, BDG measurement, and ELISA yielded results that supported a diagnosis of IPA at a mean (\pm SD) of 11.2 ± 3.5 , 11.1 ± 5.7 , 15.5 ± 8.4 , and 17.2 ± 10.2 days after the onset of fever, respectively. Positive findings of PCR preceded those of CT scan by -0.3 ± 6.6 days, those of the BDG assay by a mean (\pm SD) of 6.5 ± 4.9 days and preceded those of ELISA by a mean of 2.8 ± 4.1 days (table 2).

Typical clinical course. Figure 3 illustrates the clinical course of a patient who developed IPA with tracheobronchial involvement (patient 9, table 2) [21]. The patient died of respiratory failure caused by *Aspergillus* tracheobronchitis. ELISA, BDG measurement, and real-time PCR yielded positive results on days 165, 169, and 161 after transplantation, respectively. Real-time PCR yielded positive results earlier than did BDG assay and ELISA and reflected the response to antifungal treatment with greater sensitivity.

DISCUSSION

We developed a new method for the diagnosis of IPA, real-time PCR for use with blood samples. This method has some advantages over antigen-detection tests and conventional PCR assays.

First, real-time PCR is sensitive for IPA diagnosis. Although PCR that uses blood samples has not been successfully applied to IPA diagnosis [22], *Aspergillus* DNA was detected in 26 of 33 IPA patients in our study. The sensitivity of real-time PCR (79%) was higher than that of ELISA (58%) and that of BDG measurement (67%) and was comparable to that reported for

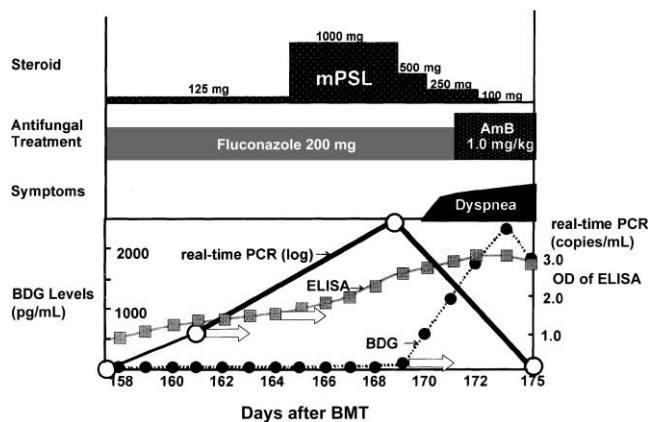


Figure 3. Clinical course of a patient who developed invasive pulmonary aspergillosis with tracheobronchial involvement after bone marrow transplantation. ELISA, BDG measurement, and real-time PCR yielded positive results on days 165, 169, and 161 after transplantation, respectively. Real-time PCR yielded positive results earlier than did the BDG assay or ELISA and reflected the response to antifungal treatment with greater sensitivity. On day 161 after transplantation, 1150 copies were detected in 1 mL of blood. The number of copies of *Aspergillus* DNA rapidly increased to 3350 copies/mL of blood on day 168. AmB, amphotericin B; BDG, (1→3)- β -D-glucan; mPSL, methylprednisolone; OD, optical density.

nested PCR [23]. Although the detection limit of real-time PCR, 20 copies/mL, may be insensitive compared with that of conventional PCR [12], we can accurately quantitate 20 copies of *Aspergillus*-specific DNA using this PCR method and can detect <10 DNA copies. Considering the quantitative ability of this assay, we defined 20 copies as its threshold.

It may be important that we extracted *Aspergillus* DNA from the blood without removal of fungal cell walls by mechanical disruption or enzymatic treatment. These steps are considered to be essential for DNA extraction from fungi. However, *Aspergillus* DNA might circulate in the blood as naked DNA or in combination with damaged cell walls. Quantitation of these DNA might be promising for fungal PCR. It is interesting that neither PCR nor the antigen detection tests uniformly had positive results more often in our study (table 3). Because the 3 assays recognize different fungal components, the combined use of these tests will improve the rate of detection of *Aspergillus* infection.

Second, real-time PCR is highly specific for *Aspergillus* infection. No *Candida* species DNA was amplified during in vitro examination, and real-time PCR yielded negative results for patients who had invasive candidiasis, *Pneumocystis carinii* pneumonia, and viral infections.

Third, real-time PCR seems to be an accurate method of quantitating fungal copies. In vitro examination showed that the CV of real-time PCR was <5% for 20–10¹⁰ *Aspergillus* copies and increased to 12.8% when the number of copies was 10. Furthermore, maximum levels (in copies/mL) for PCR corre-

lated well with those for ELISA and BDG measurement in a clinical setting (table 2). Figure 3 illustrates the clinical course of a patient who developed IPA after BMT. The number of copies found by PCR correlated well with the optical density of ELISA and levels of BDG. Real-time PCR yielded positive results earlier than the other methods and reflected the disease progress with greater sensitivity.

Real-time PCR is a promising method for use in diagnosis of IPA, but we have to comment on contamination. In *Aspergillus* PCR, contamination occurs by airborne spore inoculation during the procedures, by product carryover, and because of the presence of fungal spores in PCR materials. In this study, none of the control reagents had results that were positive for *Aspergillus* DNA, which implies that environmental contamination was negligible. Another possible cause of contamination is carryover of PCR products. PCR amplicons may serve as targets for further reactions. To avoid the carryover of amplicons, we treated PCR samples with uracil-DNA-glycosylase digestion, which has been proved elsewhere to inactivate amplicons from PCR products [24]. The other possibility is contamination of commercially available products used for PCR. These materials could be carriers of fungal spores, and many companies seem not to be aware of the fact that their products might be used for fungal PCR [25]. We also experienced such contamination of PCR reagents during the development of real-time PCR and therefore made it a rule to examine the contamination of reagents before PCR assays. Thereafter, the problem of reagent contamination disappeared.

Real-time PCR is definitely useful for IPA diagnosis, but our study has several limitations that need to be discussed. Of most importance was the finding that real-time PCR produced false-positive results more frequently than the other tests in this study. Although the in vitro study showed that real-time PCR was highly specific for *Aspergillus* species, 7 (7.8%) of 89 control patients had positive results at some point during the clinical course of their illnesses. We cannot deny the possibility that contamination was the cause of these false-positive results, but *Aspergillus*-specific DNA might have existed in these samples. We have reported elsewhere that the *Aspergillus* latex agglutination test occasionally yielded positive results in the case of neutropenic patients without IPA [10]. We discussed the possibility that some fungal components circulated in the peripheral blood of these patients and that the components were cleared with neutrophil recovery. Positive results of PCR do not necessarily indicate the presence of IPA. Real-time PCR may be helpful in differentiating true IPA from transient *Aspergillus* antigenemia, because it can provide quantitative information on the fungal burden.

Furthermore, we cannot make a definite conclusion concerning the timing of positive tests from our study. We prospectively evaluated only 15 patients with IPA. Positive findings of PCR

preceded those of CT scan by a mean (\pm SD) of -0.3 ± 6.6 days, those of the BDG assay by a mean of 6.5 ± 4.9 days, and those of ELISA by a mean of 2.8 ± 4.1 days (table 2). PCR seemed to be almost as sensitive as chest CT scan for diagnosis of IPA. How early in the clinical course PCR yields positive results for patients with IPA is an area that needs further evaluation. In conclusion, we have developed a real-time PCR for use in the diagnosis of IPA that is highly sensitive and allows us to accurately quantitate circulating copies of *Aspergillus* DNA.

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