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PCR Detection of DNA Specific for *Aspergillus* Species in Serum of Patients with Invasive Aspergillosis

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We investigated the possible presence of DNA specific for *Aspergillus* species in serum samples of patients with invasive aspergillosis (IA) by the nested PCR method. Fourteen strains of fungi including 5 strains of *Aspergillus* species and 10 strains of common bacteria were used for examination of specificity and sensitivity of the nested PCR. Two sets of oligonucleotide primers were derived from the sequence of the variable regions V7 to V9 of the 18S rRNA genes of *Aspergillus fumigatus*. The specific fragment was amplified from five strains of *Aspergillus* species in the single and nested PCR but not from other microorganisms. Target DNA was detected by the nested PCR with as little as 50 fg of the extracted DNA of *A. fumigatus*. We investigated the detection of DNA specific for *Aspergillus* species in serum samples of a murine model of aspergillosis and 20 patients with IA. The specific fragment was detected by the nested PCR in 71% of serum samples of infected mice and 70% of serum samples of patients with IA, while galactomannan antigen was detected in 43 and 60% of samples, respectively. The high sensitivity and specificity of the nested PCR indicate that the assay can provide early diagnosis with sufficient accuracy to be clinically useful for immunocompromised patients with IA.

Invasive aspergillosis (IA) is a life-threatening condition in immunocompromised patients, particularly those treated with chemotherapy for hematologic malignancies or those receiving high-dose corticosteroids (4, 5). An early diagnosis of aspergillosis is of great importance because early treatment may resolve this potentially fatal infection (1). Unfortunately, the diagnosis of IA remains difficult and sometimes is confirmed only at autopsy. At present, a firm diagnosis is established by histological examination of tissue samples obtained during open lung biopsy or transbronchial lung biopsy as well as by detecting the causative pathogenic fungi in clinical samples. Sputum cultures are unfortunately unreliable (4), and most patients are too ill to allow for lung biopsy.

Serological tests such as those involving the detection of antibodies for *Aspergillus* species are less helpful because of the poor antibody responses in immunosuppressed patients (23). In addition, the methods used for detecting circulating *Aspergillus* antigens, such as radioimmunoassay, immunoblotting assay, enzyme immunoassay, and the latex agglutination test, have poor sensitivity (14, 15). Although low levels of (1-3)-β-D-glucan can be detected directly by the G test, the sensitivity of this test is also inadequate for the early diagnosis of IA and chronic necrotizing aspergillosis (10).

Recently, PCR has been used successfully in detecting specific DNA of several pathogens (12, 19, 21). For the diagnosis of IA, PCR has been used to detect DNA specific for *Aspergillus* species in bronchoalveolar lavage (BAL) fluid from patients with IA (2, 9, 13, 17, 18). Although the sensitivity is high and the results are available within a short period of time, the BAL technique is sometimes not recommended for patients with severe underlying diseases. To our knowledge, this is the first description of the use of nested PCR to detect DNA specific for *Aspergillus* species by using serum from experimentally infected mice and patients with IA.

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MATERIALS AND METHODS

Organisms and growth conditions. The following eight strains of filamentous fungi and six strains of yeasts, supplied by Teikyo University Research Center for Medical Mycology, were used in the present study: *Aspergillus fumigatus* TIMM 0069, *Aspergillus flavus* TIMM 0058, *Aspergillus nidulans* TIMM 0111, *Aspergillus niger* TIMM 0113, *Aspergillus terreus* TIMM 0119, *Penicillium expansum* TIMM 1293, *Penicillium citreo-viride* TIMM 0882, *Penicillium crustosum* TIMM 1332, *Candida albicans* TIMM 1623, *Candida glabrata* TIMM 1064, *Candida parapsilosis* TIMM 0292, *Candida tropicalis* TIMM 0313, *Cryptococcus neoformans* TIMM 0354, and *Trichosporon beigelii* TIMM 1318.

The following 10 standard and clinical bacterial isolates were also used: *Staphylococcus aureus* MB 2786, *Staphylococcus epidermidis* and *Streptococcus pneumoniae* clinical isolates, *Klebsiella pneumoniae* ATCC 10031, *Pseudomonas aeruginosa* PAO 2001, *Escherichia coli* ATCC 39188, *Serratia marcescens* ATCC 8100, and isolates of *Haemophilus influenzae*, *Proteus mirabilis*, and *Enterobacter cloacae*.

The fungi were incubated and allowed to proliferate in Sabouraud glucose broth for 72 h at 30°C, while the common bacteria were incubated in Luria-Bertani medium for 24 hours at 37°C.

Extraction of DNA from cultured strains. Extraction of DNA from fungi was performed by a modified method of Tang et al. (18). Briefly, 50 ml of Sabouraud glucose broth in a flask was inoculated with conidia and incubated for 72 h at 30°C. The mycelial mat was transferred into a 50-ml polypropylene screw-cap tube containing six glass beads (4-mm diameter). The tube was then immersed in liquid nitrogen for 10 s and vortexed vigorously for 20 s. Then, 2 ml of DNA extraction buffer containing 10 mM Tris-HCl (pH 8.0), 10 mM EDTA, 0.15 M NaCl, 2% sodium dodecyl sulfate (SDS), and 0.5 mg of proteinase K per ml (Sigma Chemical Co., St. Louis, Mo.) was added, and the mixture was allowed to thaw. In the next step, the mixture was incubated for 2 h at 55°C. Proteinase K was then inactivated by heating the mixture to 95°C for 10 min. A volume of 0.7 ml of the mixture was transferred to a 1.5-ml microcentrifuge tube, an equal volume of phenol-chloroform (1:1) was added, and the mixture was centrifuged at 10,000 × *g* for 5 min at 4°C (MRX-150; Tomy Seiko Co., Tokyo, Japan). The supernatant was transferred to a fresh tube, and the same procedure was repeated with chloroform-isoamyl alcohol (24:1). The DNA was precipitated with 2 volumes of ethanol at −20°C and centrifuged at 12,000 × *g* for 20 min at 4°C, and the pellet was allowed to dry. After rinsing with 70% ethanol at 4°C, the extracted DNA was dissolved in 50 μl of distilled water and 5 μl of suspension was used for PCR.

Extraction of DNA from the common bacteria was performed according to the method using lysis by alkali and SDS, as described by Sambrook et al. (16).

Clinical samples and DNA extraction. We evaluated 20 patients with a histological diagnosis of IA and 20 healthy volunteers. Blood samples were centrifuged at 2,500 × *g* for 10 min at 4°C. The sera were either processed immediately or stored at −20°C until use. Extraction of DNA from sera was performed according to the method described by Tokimatsu et al. (19). In the first step, a serum sample of 100 μl was combined with the same volume of lysis buffer

containing 100 mM KCl, 20 mM Tris-HCl (pH 8.3), 5 mM $MgCl_2$, 0.2 mg of gelatin per ml, and 0.9% polysorbate 20 (Tween 20) solution. Proteinase K was added to a final concentration of 60 μg/ml. The mixture was incubated for 60 min at 55°C. Proteinase K was then inactivated by heating the mixture to 95°C for 10 min. The supernatant was used for PCR amplification following centrifugation at 12,000 × *g* for 10 min at 4°C.

Oligonucleotide primers and PCR. The design of oligonucleotides used in this study was based on comparison of the sequence of 18S rRNA genes of *Aspergillus* species and other fungi in the GenBank database. The PCR was performed as a nested PCR with two sets of primers. The outer primer set consisted of Asp. 5 (5′GATAACGAACGAGACCTCGG3′) and Asp. 8 (5′TGCCAACTCCCCTG AGCCAG3′), amplifying a 384-bp sequence. The inner primer set consisted of Asp. 1 (5′CGGCCCTTAAATAGCCCGGTC3′) and Asp. 7 (5′CCTGAGCCA GTCCGAAGGCC3′), amplifying a 357-bp sequence.

The reaction mixtures used in the present series of experiments consisted of 50 mM KCl, 10 mM Tris-HCl (pH 8.8), 1.5 mM $MgCl_2$, 0.1% octoxynol (Triton X-100), 160 μM (each) four deoxynucleotide triphosphates (dATP, dCTP, dTTP, and dGTP), and 1 U of *Thermus aquaticus* (*Taq*) DNA polymerase (Takara Taq; Takara Shuzo Co., Otsu, Japan). In a single PCR step, 50 pmol of each outer primer was combined with 5 μl of the prepared sample to a final volume of 50 μl. The tube was covered with mineral oil to prevent evaporation, and PCR was conducted in an automatic thermal cycler (program temperature control system PC-700; Astec Co., Fukuoka, Japan). The amplification reactions were run for 30 cycles of DNA denaturation at 94°C for 1 min, primer annealing at 50°C for 1 min, and DNA extension at 72°C for 3 min. The final cycle of PCR extension was carried out at 72°C for 10 min. In the nested PCR step, 1.0 μl of the product from the first amplification was added to a new reaction mixture with 50 pmol of each inner primer and reamplified with 30 cycles as described above except for the primer annealing step. This step was carried out at 65°C for 1 min. To avoid possible contamination of PCR mixtures, all reactions were performed under stringent conditions as recommended by Kwok and Higuchi (7). Three negative controls, including reagent controls and sera from healthy volunteers, were run along with the test samples for all reactions. The nested PCR products were electrophoresed on a 2% agarose gel containing ethidium bromide, and the results were photographed.

Southern blot hybridization. Gel-fractionated nested PCR products were transferred to nylon membranes (Hybond N^+; Amersham International, Amersham, England) and then hybridized with the Asp-p probe (5′ATGGAAGTGC GCGGCAATAAC3′) (9) labeled with a chemiluminescence detection system (ECL 3′ oligolabeling and detection system; Amersham, Little Chalfont, Buckinghamshire, United Kingdom). The membranes were washed according to the instructions provided by the manufacturer and exposed to X-ray films for 30 min.

Galactomannan assay. Circulating galactomannan antigen was detected by a latex agglutination test, Pastorex Aspergillus (Diagnostics Pasteur, Marnes-la-Coquette, France). Assays were performed according to the instructions provided by the manufacturer. Briefly, the serum sample was incubated at 100°C with a serum treatment solution for 3 min. After centrifugation, the supernatant was tested with latex beads.

Animal model of aspergillosis. Animal studies were performed according to the guidelines of the Ethical Committee for Animal Experimentation released by Oita Medical University. We established an animal model of aspergillosis with *A. fumigatus* as described previously (3, 9, 11) with some modifications. Briefly, with 12 male ICR mice (8 weeks old; Charles River Japan), each animal was immunosuppressed by three consecutive intraperitoneal doses of cyclophosphamide (150 mg/kg of body weight) administered on days −4, −3, and −2 followed by three consecutive doses of prednisolone (30 mg/kg, intraperitoneally) on days −1, 0, and +1. On day 0, introduction of the microorganisms was performed with eight animals under ketamine anesthesia (Sankyo Co., Tokyo, Japan). For this purpose, an incision was made over the trachea, and 30 μl of inoculum containing 10^6 viable conidia was inoculated transtracheally. Tetracycline HCl (Sigma; 1.0 g/liter) was added to the drinking water to prevent bacterial infection.

Two infected mice and one control were sacrificed under anesthesia with ether on days +1, +2, +3, and +5. Following dissection, several blood samples were obtained through a puncture of the heart. A volume of 50 μl of each blood sample was cultured on Sabouraud dextrose agar at 30°C for 7 days. The remaining blood samples were used for PCR and galactomannan assays according to the methods described above. Sections from both lungs, liver, spleen, and both kidneys were fixed in buffer formalin solution, embedded in paraffin, and later stained with hematoxylin and eosin, Gomori methenamine silver stain, and periodic acid-Schiff technique to confirm the presence of aspergillosis.

RESULTS

Specificity of nested PCR for *Aspergillus* species. DNA samples from the above organisms were tested to see whether the primers amplified the same DNA products. By a single PCR with outer primers, a specific 384-bp fragment was amplified from all tested *Aspergillus* species, i.e., *A. fumigatus*, *A. flavus*, *A. nidulans*, *A. niger*, and *A. terreus*, but not from other microorganisms, including *P. citreo-viride*, *P. expansum*, and *P. crustosum*. Similarly, by nested PCR with inner primers, a specific 357-bp fragment was amplified from the above *Aspergillus* species but not from other microorganisms. This specificity was confirmed following Southern blot hybridization (Fig. 1 and 2).

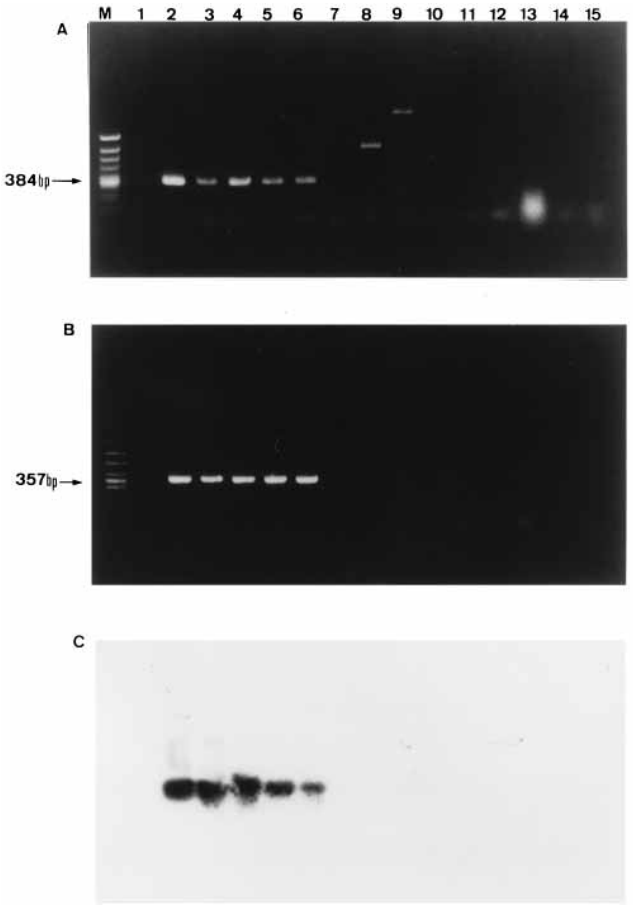

FIG. 1. Specificity of the PCR assay with DNA from various pathogenic fungi. (A) Single PCR assay; (B) nested PCR assay; (C) Southern blot hybridization. Lanes: M, molecular size marker (ϕ174/*Hin*cII digest); 1, distilled water; 2, *A. fumigatus*; 3, *A. flavus*; 4, *A. nidulans*; 5, *A. niger*; 6, *A. terreus*; 7, *Candida albicans*; 8, *Candida glabrata*; 9, *Candida parapsilosis*; 10, *Candida tropicalis*; 11, *Cryptococcus neoformans*; 12, *T. beigelii*; 13, *P. citreo-viride*; 14, *P. expansum*; 15, *P. crustosum*.

Sensitivity of nested PCR for *Aspergillus* species. We used an *A. fumigatus* gene, extracted as described above, to evaluate PCR sensitivity. The gene was serially diluted by 10-fold, and each dilution was then subjected to amplification by PCR. Fifty picograms of target DNA was detected by the single PCR, whereas as little as 50 fg could be detected by the nested PCR, indicating that the sensitivity of the nested PCR was 1,000 times higher than that of the single PCR. The sensitivity of Southern blot hybridization was 10 times higher than that of the nested PCR (Fig. 3).

Animal model of IA. The results of blood cultures, histological findings, galactomannan antigen, and nested PCR are summarized in Table 1. Blood cultures of all eight infected mice were negative for *A. fumigatus*, although histological evidence of aspergillosis was found in seven of eight mice. Both the nested PCR and galactomannan antigen became positive on day 1; however, the PCR showed a higher positive rate (71%) than galactomannan antigen (43%) in histologically confirmed IA. In one mouse, there was no histological evidence of IA,

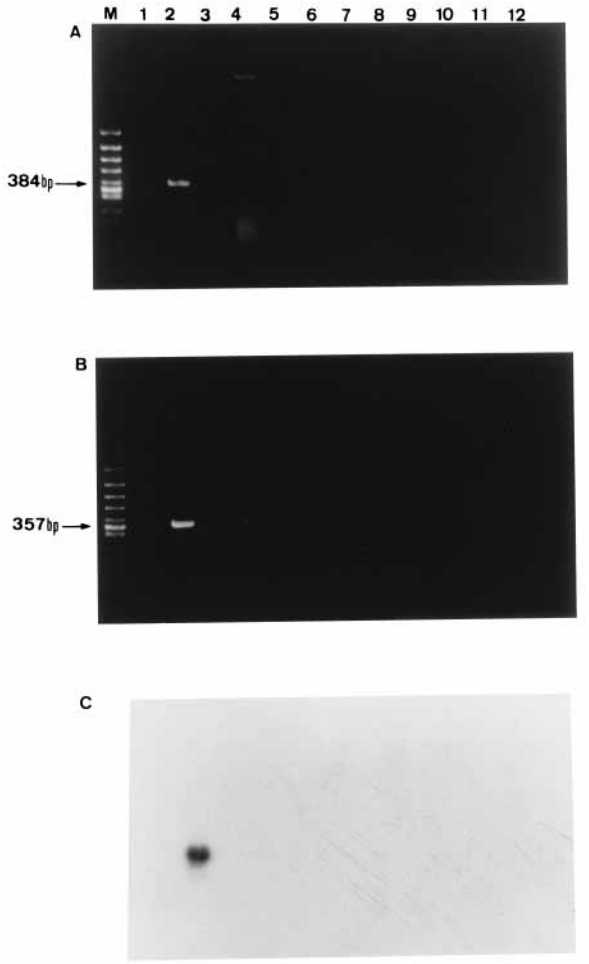

FIG. 2. Specificity of the PCR assay with DNA from various common bacteria. (A) Single PCR assay; (B) nested PCR assay; (C) Southern blot hybridization. Lanes: M, molecular size marker (ϕ174/*Hinc*II digest); 1, distilled water; 2, *A. fumigatus*; 3, *Staphylococcus aureus*; 4, *Staphylococcus epidermidis*; 5, *Streptococcus pneumoniae*; 6, *H. influenzae*; 7, *K. pneumoniae*; 8, *Pseudomonas aeruginosa*; 9, *Escherichia coli*; 10, *Serratia marcescens*; 11, *Proteus mirabilis*; 12, *Enterobacter cloacae*.

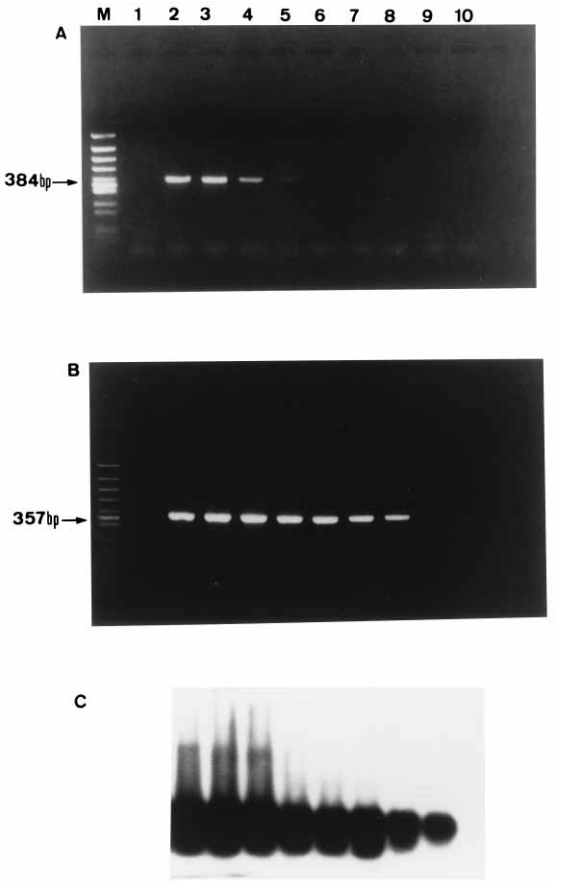

FIG. 3. Sensitivity of the PCR assay with DNA from *A. fumigatus*. (A) Single PCR assay; (B) nested PCR assay; (C) Southern blot hybridization. Lanes: M, molecular size marker (ϕ174/*Hinc*II digest); 1, distilled water; 2, 5×10^{-8} g; 3, 5×10^{-9} g; 4, 5×10^{-10} g; 5, 5×10^{-11} g; 6, 5×10^{-12} g; 7, 5×10^{-13} g; 8, 5×10^{-14} g; 9, 5×10^{-15} g; 10, 5×10^{-16} g.

and the results of nested PCR and galactomannan antigen were negative. There was no histological or culture evidence of *A. fumigatus* infection in all immunosuppressed control mice, and the nested PCR was also negative in all cases.

Detection of DNA specific for *Aspergillus* species in clinical samples. A summary of the results of nested PCR and galactomannan antigen assay in serum samples from patients with a histological diagnosis of IA is shown in Table 2. All patients had negative blood cultures. The nested PCR was positive for 14 of 20 patients (70%), while galactomannan antigen was positive for 12 (60%). All sera from healthy volunteers were negative for the nested PCR and galactomannan antigen assay.

DISCUSSION

We have demonstrated for the first time the usefulness of PCR for detecting DNA specific for *Aspergillus* species in sera of mice serving as a model of aspergillosis and clinical samples from patients with the same mycotic disease. The method is suitable for immunocompromised patients with severe underlying diseases because serum samples can be obtained easily. Several investigators have reported recently the use of PCR in detecting DNA specific for *Aspergillus* species in BAL fluids (2, 9, 13, 17, 18). However, to our knowledge, there is no report describing the use of PCR for detecting *Aspergillus* DNA in the sera of patients with IA. While the risk of performing BAL is

TABLE 1. Detection of *A. fumigatus* in serum of experimentally infected mice

No. of days after infection	Mouse no.	Result of test[a]:			
		Blood culture	Histology	Galactomannan antigen	Nested PCR
1	1	−	+	+	+
	2	−	−	−	−
2	3	−	+	−	−
	4	−	+	+	+
3	5	−	+	+	+
	6	−	+	−	+
4	7	−	+	−	−
	8	−	+	−	+
Control	C1	−	−	−	−
	C2	−	−	−	−
	C3	−	−	−	−
	C4	−	−	−	−

[a] −, negative; +, positive.

TABLE 2. Comparison between the results of nested PCR and galactomannan assays using serum samples from patients with histologically diagnosed IA

Patient no.	Sex[a]	Age (yr)	Underlying disease[b]	Result of test:	
				Galactomannan antigen[c]	Nested PCR[d]
1	M	59	Malignant lymphoma	2+	+
2	M	29	ALL	2+	+
3	F	58	AML	1+	+
4	M	83	ALL	1+	−
5	F	50	AML	2+	−
6	F	81	Rectal cancer	1+	+
7	M	47	AML	1+	+
8	M	72	Pulmonary asbestosis	2+	+
9	M	53	Pulmonary tuberculosis	2+	+
10	F	22	SLE	2+	+
11	M	28	AML	1+	+
12	M	41	AML	2+	+
13	M	73	Malignant lymphoma	−	−
14	M	60	Pneumoconiosis	−	−
15	F	32	MDS	−	+
16	M	75	IIP	−	−
17	F	53	Malignant lymphoma	−	+
18	M	46	ATL	−	−
19	M	48	AML	−	+
20	M	71	IIP	−	+

[a] M, male; F, female.
[b] ALL, acute lymphoblastic leukemia; AML, acute myeloblastic leukemia; SLE, systemic lupus erythematosus; MDS, myelodysplastic syndrome; IIP, idiopathic interstitial pneumonia; ATL, adult T-cell leukemia.
[c] −, no agglutination; 1+, the degree of latex agglutination with sample was as great as that with positive control; 2+, the degree of latex agglutination with sample was greater than that with positive control.
[d] +, positive; −, negative.

lower than that of open lung biopsy or transbronchial lung biopsy in immunocompromised patients, its use is still limited and it cannot be performed repeatedly in such patients, whereas blood samples can be withdrawn comparatively easily. Detection of DNA specific for *Aspergillus* species by our method is very useful because it is noninvasive and can be repeated several times. The sensitivity of the nested PCR in detecting DNA specific for *Aspergillus* species in patients with IA was 70%, indicating that the assay is clinically useful.

The novel primers used in this study were highly sensitive and specific. IA is usually caused by *A. fumigatus* or *A. flavus* (18), but infection due to other species is not uncommon (6, 22). In this study, we developed novel primers to detect not only *A. fumigatus* and *A. flavus* but also other clinically relevant DNA specific for *Aspergillus* species by nested PCR. When molecular biological techniques are used for the detection of *Aspergillus* species, one must consider a possible cross-reaction between *Aspergillus* species and *Penicillium* species (8, 9). The novel primers used in the present experiments did not amplify DNA from three strains of *Penicillium* species. Unfortunately, we were unable to examine *Penicillium marneffei*, which causes opportunistic infection in patients with AIDS. In this regard, there is a strong close homology in the sequence alignment in the variable regions V7 to V9 of the 18S rRNA genes between *Aspergillus* species and *P. marneffei*. Spreadbury and colleagues (17) also reported a close homology between the two on the 26S rRNA sequence. While we admit that such a possibility may exist, in this case, Southern blot hybridization using the DNA probe Asp-p (9) was helpful in differentiating between *Aspergillus* species and *P. marneffei*.

An early diagnosis of IA is critical for a successful treatment. Recent studies demonstrated that early diagnosis of IA can be achieved through the detection of galactomannan antigen (10). The present results extended these early findings by demonstrating the presence of galactomannan antigen as well as a positive nested PCR 1 day after inoculation of the microorganism in our experimental murine model of aspergillosis. On the basis of these findings, we consider that early diagnosis can be attained by the presence of a positive nested PCR and/or galactomannan antigen. Our results further demonstrated that the sensitivity of the nested PCR was higher than that of galactomannan antigen. Thus, 71% of mice with a histological diagnosis of IA were positive for nested PCR, while only 43% of the same animals were positive for galactomannan antigen. In one mouse, no histological evidence of IA was present. This was probably due to unsuccessful induction of infection. In two other mice, nested PCR and galactomannan assay were not positive in spite of histological evidence of IA. These results may be explained by the development of only a mild form of infection or early clearance of *A. fumigatus* from the lung. Our results further demonstrated no false-positive cases by both assays. On the basis of these findings, the nested PCR provides sufficient sensitivity and specificity for detecting DNA specific for *Aspergillus* species in experimental murine aspergillosis.

The clinical results by the same assays were similar to those of animal studies. DNAs specific for *Aspergillus* species were detected by the nested PCR in 14 of 20 patients (70%) with histologically confirmed IA. The patients included those with hematological malignancies and patients with pulmonary diseases treated with corticosteroids between 1983 and 1995 at Oita Medical University Hospital. The blood samples were obtained from these patients and stored at −20°C. The sensitivity of the nested PCR was higher than that of the galactomannan assay. Four of six false-negative nested PCR samples were technically different from the remaining samples, as they were stored for more than 5 years and were frozen and thawed several times until used in the assay. In two other samples, the false-negative PCR may be related to a mild form of aspergillosis since the disease was not the direct cause of death. On the basis of the sensitivity and specificity of the nested PCR, the test can provide early diagnosis with sufficient accuracy to be clinically useful in immunocompromised patients with IA. A recent study by Verweig et al. (20) demonstrated that the sandwich enzyme-linked immunosorbent assay (ELISA), which detects galactomannan antigen in serum samples, is also useful for the early diagnosis of IA. While we could not compare the nested PCR with the sandwich ELISA in the present study, we believe it may be useful to combine the two tests to improve sensitivity and specificity since the two assays have different targets.

The nested PCR has several inherent problems as well as technical considerations specific to aspergillosis. The first such problem relates to possible DNA contamination. However, there was no contamination in this study since the control samples employed in every assay, comprising 5 to 10 samples, were always negative. Environmental contamination is another serious problem since *Aspergillus* species are present in the environment. However, none of the control reagents and serum samples of healthy volunteers that were tested along with the test samples were positive, indicating that if environmental contamination does occur, it is less than the threshold of this assay (18). In this regard, serum is a suitable biological sample for the nested PCR to minimize false-positive results since no organisms are usually isolated from these samples.

In conclusion, we were successful in detecting DNA specific for *Aspergillus* species in serum samples from animal models of aspergillosis and patients with IA by using the nested PCR.

Although we consider this method very useful for the diagnosis of IA, we believe that the diagnostic test needs further prospective evaluation with a large clinical population sample.

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