Comparison of PCR, (1→3)- β -D-Glucan and Galactomannan Assays in Sera of Rats With Experimental Invasive Aspergillosis

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We compared PCR, galactomannan detection assay using a latex agglutination test and (1→3)- β -D-glucan detection assay in detecting infection in rats experimentally infected with *Aspergillus fumigatus*. On day 2 after inoculation, (1→3)- β -D-glucan and nested PCR were positive for 80%, while galactomannan detection assay was positive for 60%. In addition, the positive result of nested PCR (87.5%) was higher than those of galactomannan detection assay (75%) and (1→3)- β -D-glucan (71.4%) on day 3 after inoculation. The sensitivity of nested PCR was superior to those of galactomannan detection assay

and (1→3)- β -D-glucan detection assay. The three diagnostic tests were compared with histopathological findings, and the sensitivity of three diagnostic tests was correlated with histopathological changes. In addition, the elevated levels of (1→3)- β -D-glucan paralleled the development and progression of pulmonary aspergillosis. Our results indicate that a combination of two or three of these tests seems to provide a rapid diagnosis of invasive aspergillosis and assist in the evaluation of the development and severity of invasive aspergillosis. J. Clin. Lab. Anal. 12:257–262, 1998. © 1998 Wiley-Liss, Inc.

Key words: β -glucan; galactomannan assay; aspergillosis

INTRODUCTION

Invasive aspergillosis (IA) is a very severe opportunistic fungal infection in cancer patients, particularly in patients with cytotoxic drug-induced neutropenia and those on a high-dose of corticosteroids (1,2). Early diagnosis of IA is often difficult, and a firm diagnosis is established after histological examination of tissue material such as open lung biopsy or transbronchial lung biopsy (TBLB) or detection of fungi in clinical samples. Sputum cultures are unfortunately unreliable and invasive approaches are somewhat limited.

Several laboratory methods have been tested lately for the early and rapid diagnosis of IA such as radioimmunoassay (RIA) (3–6), counter immunoelectrophoresis (CIE) (7), immunoblotting assay, enzyme immunoassay (EIA) (3,8,9), latex agglutination test (10), (1→3)- β -D-glucan detection assay (11) and polymerase chain reaction (PCR) (12–16). However, tests aimed at detecting *Aspergillus* cell-wall antigens have low sensitivity (17,18), and (1→3)- β -D-glucan detection assay has a low specificity (19). Recent advances in molecular biology have allowed the use of new techniques in clinical diagnostic microbiology (20–23). A genetic approach to the diagnosis of IA by PCR has been reported, which was used successfully for detecting specific DNA of *Aspergillus* species in bronchoalveolar lavage (BAL) fluid (12–16). Although PCR is a rapid and highly sensitive method, BAL technique is sometimes invasive particularly in patients with severe underlying diseases.

We have developed recently a nested PCR technique to detect specific DNA of *Aspergillus* species in the serum (24). This method has a high sensitivity and specificity, and can be used in patients with severe underlying diseases such as immunocompromised patients.

In this study, we extended our early investigations by comparing the nested PCR with other tests, including galactomannan detection assay, using a latex agglutination test, and (1→3)- β -D-glucan detection assay, in detecting infection caused by *Aspergillus fumigatus*.

MATERIAL AND METHODS

Organism

A. fumigatus strain IFM 41944 was cultured on Sabouraud glucose agar plates for 72 hr at 30°C. The conidia were harvested using 0.02% Tween 80–0.9% NaCl. After washing twice in 0.9% NaCl, the conidia were suspended in 0.9% NaCl and then counted in a hemocytometer. Hemocytometer count

Grant sponsor: Ministry of Education, Science and Culture, Japan; Grant number: C08670670.

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Received 15 October 1997; Accepted 5 December 1997

was confirmed by culturing serial dilutions of the inoculum size on Sabouraud glucose agar at 30°C for 72 h.

Animal Model

Animal studies were performed according to the Guidelines of the Ethics Committee for Animal Experimentation at Oita Medical University. A murine model of infection with *A. fumigatus* was established as previously described (25), with few modifications. Briefly, a total of 24 male Sprague Dawley rats (8-week-old, Charles River, Japan) were immunosuppressed by an intraperitoneal (i.p.) injection of 200 mg/kg cyclophosphamide (Shionogi Co., Osaka, Japan) on day -3 followed by three consecutive doses of prednisolone (20 mg/kg, i.p.) on days -2, -1 and 0. On day 0, *A. fumigatus* was instilled in 22 animals under ketamine anesthesia (Sankyo Co., Tokyo, Japan). For this purpose, an incision was made over the trachea, and 30 µl of inoculum containing 5×10^6 viable conidia was inoculated transtracheally. Vancomycin (50 µg/ml) and gentamicin (10 µg/ml) were added to drinking water to prevent bacterial infection. Control rats received exactly the same treatment except for inoculation of the conidia.

Five infected rats were sacrificed under anesthesia with ether on days +1 and +2, eight on day +3, and two on days +4 and +5, and two control rats were sacrificed under anesthesia on day +5. Blood samples were obtained by cardiocentesis. A volume of 50 µl of blood sample was cultured on Sabouraud glucose agar at 30°C for 4 days. The serum was then used for (1→3)-β-D-glucan assay, nested PCR and galactomannan detection. Tissue sections from both lungs, liver, spleen, and both kidneys were fixed in buffer formalin solution and embedded in paraffin stained with hematoxylin and eosin, Gomori methenamin silver stain, and periodic acid-Schiff technique to confirm the inflammation and the presence of the fungus.

PCR Assay

The design of oligonucleotides used in this study was based on comparison of the sequences of 18S ribosomal RNA genes (rDNA) of *A. fumigatus*. Nested PCR was performed according to the method described by Yamakami et al. (24). Briefly, the outer primer set consisted of 5'-GATAACGAACGAGACCTCGG-3' and 5'-TGGCTCAGGGGAGTTGGCA-3' amplifying a 384 base pair sequence. The inner primer set consisted of 5'-CGGCCCTTAAATAGCCCGGTC-3' and 5'-GGCCTTCGGACTGGCTCAGGG-3' amplifying a 357 base pair sequence. The amplification reactions of single PCR 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. The nested PCR step was reamplified with 30 cycles as described above except for the primer annealing step, which was performed at 65°C for 1 min.

Nested PCR products were electrophoresed on 2% of agarose gel containing ethidium bromide and the results photographed. Southern blot hybridization was performed with the Asp-p probe (5'-ATGGAAGTGCGCGGCAATAAC-3') described previously by Melchers et al. (13).

(1→3)-β-D-Glucan Assay (M-0198)

The reagent for (1→3)-β-D-glucan determination assay (M-0198) was kindly provided by Maruha Corp. (Tsukuba, Japan). M-0198 reagent contains factor G, a horseshoe crab coagulation enzyme that is extremely sensitive and specific to (1→3)-β-D-glucan. The assay was performed as previously described (26). A serum sample was fourfold diluted and incubated at 70°C for 10 min. After cooling, the sample was added to M-0198 reagent and incubated at 37°C for 30 min. Absorbance at 545 and 630 nm was measured after diazocoupling. Samples with concentrations exceeding 50 pg/ml were considered positive for *Aspergillus*.

Galactomannan Assay (Pastorex)

The presence of circulating galactomannan antigen was detected using a latex agglutination test, Pastorex Aspergillus (Diagnostics Pasteur, Marnes-la-Coquette, France), in which latex beads are coated with an antigalactomannan monoclonal antibody. Assays were performed according to the instructions provided by the manufacturer. Thus, serum samples were incubated at 100°C with the serum treatment solution for 3 min. After centrifugation, the supernatants were tested with the latex beads. The presence of an antigen titer of 15 ng/ml represented a positive agglutination reaction.

RESULTS

Table 1 shows the results of blood culture, pathological findings, galactomannan assay, (1→3)-β-D-glucan assay, and nested PCR. In rat 13, (1→3)-β-D-glucan was not performed because a little volume of blood sample was obtained.

Pathological and Mycological Examination

The histological sections were examined for the presence of *A. fumigatus* and determination of the histopathological changes. The fungus was only detected in the lungs (Figs. 1–3). In rat 4, the conidia and hyphae were found confined to the mucosal surface of the bronchi but there was no clear inflammatory response (Fig. 1). In rat 9, marked changes were observed, including hyphal invasion of the bronchial walls, and fungal bronchopneumonia characterized by peribronchial inflammation and extension of the inflammatory infiltrate into the alveoli (Fig. 2). In rat 16, hyphal invasion was more advanced to include blood vessels, resulting in hemorrhagic areas scattered throughout the microscopic fields (Fig. 3). The histopathological changes were graded based on the following three parameters: + represented bronchial

TABLE 1. Detection of *Aspergillus fumigatus* in Sera of Experimentally Infected Rats

Rat	Days after inoculation	Blood Culture	Histo-pathology	Pastorex* <i>Aspergillus</i>	β -glucan ¶ (pg/ml)	Nested PCR
1	Control	—	—	—	0	—
2	Control	—	—	—	0	—
3	1	—	±	+	42	+
4	1	—	+	—	128	—
5	1	—	±	—	40	—
6	1	—	+	—	5	—
7	1	—	±	—	12	—
8	2	—	+	+	80	+
9	2	—	++	+	294	+
10	2	—	+	—	52	+
11	2	—	++	+	0	+
12	2	—	±	—	100	—
13	3	—	+++	+	NP	+
14	3	—	+	—	112	+
15	3	—	++	+	420	+
16	3	—	+++	+	467	+
17	3	—	+++	+	315	+
18	3	—	+	+	138	+
19	3	—	+	+	39	+
20	3	—	+	—	6	—
21	4	—	++	+	249	+
22	4	—	+++	+	427	+
23	5	—	++	+	85	+
24	5	—	++	+	36	—

Histopathological grading: +, bronchial involvement; ++, invasion of the bronchial wall; +++, presence of vascular invasion. *, Pastorex positive > 15 ng/ml; ¶, β -glucan positive > 50 pg/ml. NP, not performed; PCR, polymerase chain reaction.

involvement, ++ invasion of the bronchial wall, and +++ denoted the presence of vascular invasion (Table 1).

All blood cultures of infected rats and two control rats were negative for *A. fumigatus*.

PCR Assay

On day 1 after inoculation, nested PCR was positive in one rat but negative in others. On day 2 after inoculation, nested PCR was positive in four of five rats (80%), and seven of eight rats examined on day 3 had positive PCR (87.5%). On day 4 after inoculation, nested PCR were positive in both rats. Nested PCR was negative in two immunosuppressed control rats (Table 1).

(1→3)- β -D-Glucan Assay (M-0198)

One day after inoculation, (1→3)- β -D-glucan concentration was 128 pg/ml in rats 4, and below 50 pg/ml in the others (Table 1). On day 2 after inoculation, the concentration of (1→3)- β -D-glucan was positive in four of five rats (80%). On day 3 after inoculation, the concentration of (1→3)- β -D-glucan was over 50 pg/ml in five of seven rats (71.4%). On day 4 after inoculation, the concentrations of (1→3)- β -D-glucan were 249 and 427 pg/ml in rats 21 and 22, respectively. Examination was not performed in one infected rat sacrificed 3 days postinoculation. The test was negative in two immunocompromised control rats (Table 1).

Galactomannan Assay (Pastorex)

On day 1 after inoculation, the galactomannan antigen detection assay (Pastorex, Table 1) was positive in one rat and negative in others (20%). On day 2, galactomannan antigen was positive in three of five infected rats (60%). Pastorex was positive in six of eight infected rats (75%), and both rats on day 4 had positive Pastorex (100%). Both control rats had a negative Pastorex.

DISCUSSION

The early diagnosis and treatment of IA is a key determinant of a successful outcome (1,2). Several methods that provide early and rapid diagnosis of IA are currently being investigated (3–16). In this study, we compared nested PCR, galactomannan detection assay using a latex agglutination test and (1→3)- β -D-glucan detection assay in rats experimentally infected with *A. fumigatus*.

In our rat model of experimental aspergillosis, the three diagnostic methods were compared on a daily basis (Table 2). One day after inoculation, the results of galactomannan detection assay and nested PCR were positive only in rat 3, while (1→3)- β -D-glucan detection assay was positive only in rat 4. The small population of these serodiagnostic methods makes comparison of the sensitivity of them very difficult at early stage of infection. On day 2 after inoculation, (1→3)- β -D-glucan and nested PCR were positive for 80%, while

Fig. 1. Histopathological features of a representative example of a lung specimen obtained from sacrificed rat 4. Conidia and hyphae are confined to the mucosal surface of the bronchi, without any inflammatory response (histopathological grading +). **Top:** Hematoxylin and eosin staining. Magnification, $\times 200$. **Bottom:** Gomori methenamine silver stain. Magnification, $\times 200$.

galactomannan detection assay was positive for 60%. In addition, the positive result of nested PCR (87.5%) was higher than those of galactomannan detection assay (75%) and (1 $\rightarrow$ 3)- β -D-glucan (71.4%) on day 3 after inoculation. The negative galactomannan detection assay and nested PCR in rat 12 on day 2 and rat 20 on day 3 after inoculation were probably due to the presence of a mild-to-moderate degree of pulmonary aspergillosis, where conidia and hyphae were confined to the mucosal surface of the bronchi with no evidence of an inflammatory response. We have previously shown the superiority of PCR sensitivity to galactomannan detection assay (24). The results of this study demonstrated that the sensitivity of nested PCR was superior to those of galactomannan detection assay and (1 $\rightarrow$ 3)- β -D-glucan detection assay.

Recently, a direct double sandwich enzyme-linked immunosorbent assay has been developed (27,28). This assay employs the rat monoclonal antibody EB-A2 to detect

Fig. 2. Histopathological features of a representative example of a lung specimen obtained from sacrificed rat 9. Note hyphal invasion through bronchial wall, and fungal bronchopneumonia evident by peribronchial inflammation and extension of the inflammatory infiltrate into the alveoli (histopathological grading ++). **Top:** Hematoxylin and eosin staining. Magnification, $\times 100$. **Bottom:** Gomori methenamine silver stain. Magnification, $\times 100$.

galactomannan, a cell wall component of *Aspergillus* spp. Although the galactomannan detection assay using a latex agglutination test was used in the present study, the sensitivity of a new detection assay may be superior to that of the latex agglutination test because of the low detection limit of the formers (1ng). However, both these assays cannot detect some species relevant to cause invasive aspergillosis.

In our rat model of experimental aspergillosis, the three diagnostic methods were compared on histopathological basis (Table 3). At the early stage of infection, conidia and hyphae were present in the lung but confined to the mucosal surface of the bronchi in eight tested rats (histopathological grading +). At that stage, (1 $\rightarrow$ 3)- β -D-glucan and nested PCR were positive for five of eight rats (63%), while galactomannan detection assay was positive for three (38%). The results of galactomannan detection, (1 $\rightarrow$ 3)- β -D-glucan and nested PCR were 100%, 67%, and 83% in six infected rats with hyphal

Fig. 3. Histopathological features of a representative example of a lung specimen obtained from sacrificed rat 16. Note increased hyphal invasion into the local blood vessels and hemorrhage (histopathological grading +++). **Top:** Hematoxylin and eosin staining. Magnification, $\times 100$. **Bottom:** Gomori methenamine silver stain. Magnification, $\times 100$.

invasion through bronchial walls and peribronchial inflammation (histopathological grading ++). In the presence of vascular invasion, the results of the three diagnostic tests were all positive (histopathological grading +++). The negative (1 $\rightarrow$ 3)- β -D-glucan detection assay in rat 11 on day 2 after inoculation was probably due to technical failure because the level of (1 $\rightarrow$ 3)- β -D-glucan was 0 pg/ml. The results of this study demonstrated that the sensitivity of three diagnostic tests was correlated with histopathological changes.

Interestingly, the serum concentrations of (1 $\rightarrow$ 3)- β -D-glucan were correlated with histopathological changes (Table 1). High

TABLE 2. Comparative Results of the Three Diagnostic Tests in Time Course

Day after inoculation	1	2	3	4	5
Number of rats	5	5	8	2	2
Pastorex <i>Aspergillus</i>	20%	60%	75%	100%	100%
(1 $\rightarrow$ 3)- β -D-glucan	20%	60%	75%	100%	100%
Nested PCR	20%	80%	87.5%	100%	50%

(1 $\rightarrow$ 3)- β -D-glucan levels are known to correlate with the extent of aspergillosis (11). Our results confirm that the elevated levels of (1 $\rightarrow$ 3)- β -D-glucan paralleled the development and progression of pulmonary aspergillosis. However, the specificity of (1 $\rightarrow$ 3)- β -D-glucan detection assay is poor because it is also positive in other fungal infections (19). Quantitative analysis by the dilution method may allow nested PCR and galactomannan detection to reflect the degree of infection. Previous studies have shown that elevation of galactomannan antigen titers also paralleled the severity of the disease in experimental invasive aspergillosis (10).

Nested PCR and (1 $\rightarrow$ 3)- β -D-glucan detection assays are time consuming and labor intensive compared with the galactomannan detection assay. In addition, a major disadvantage of nested PCR is contamination. We were very careful to prevent it in this study, and that no such contamination occurred was evident by the high correlation between histopathological findings as well as serodiagnostic tests and the results of the nested PCR. Considered together, we recommend that two or more of these diagnostic tests be used in combination to provide early diagnosis of IA and evaluate the severity of infection.

In conclusion, we believe that although these methods are very useful for the diagnosis of IA, their utility should be further evaluated in a prospective fashion in a large number of patients. Such work is underway in our laboratory.

ACKNOWLEDGMENTS

We thank Kiyoko Arita for preparing fungal strains and Takako Sato for performing galactomannan detection assay. We also thank Dr. F. Issa (Department of Medicine, University of Sydney, Sydney, Australia) for the careful reading and editing of the manuscript.

This work was supported in part by a grant-in-aid for scientific research (C) (08670670) from the Ministry of Education, Science, and Culture, Japan.

TABLE 3. Comparative Results of the Three Diagnostic Tests With Histopathological Findings

Group	Histopathology	Pastorex <i>Aspergillus</i>	(1 $\rightarrow$ 3)- β -D-glucan	Nested PCR
Control	–	0/2 (0%)	0/2 (0%)	0/2 (0%)
	±	1/4 (25%)	1/4 (25%)	1/4 (25%)
	+	3/8 (38%)	5/8 (63%)	5/8 (63%)
Infection	++	6/6 (100%)	4/6 (67%)	5/6 (83%)
	+++	4/4 (100%)	3/3 (100%)	4/4 (100%)

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