EUROPEAN JOURNAL OF Haematology

European Journal of Haematology 90 (331–339)

ORIGINAL ARTICLE

Clinical utility of a panfungal polymerase chain reaction assay for invasive fungal diseases in patients with haematologic disorders

Yumiko Sugawara[1], Kazunori Nakase[2], Akiko Nakamura[3], Kohshi Ohishi[4], Yuka Sugimoto[1], Atushi Fujieda[1], Fumihiko Monma[1], Kei Suzuki[1], Masahiro Masuya[1], Yoshiko Matsushima[3], Hideo Wada[3], Tsutomu Nobori[3], Naoyuki Katayama[1]

[1]Department of Hematology and Oncology, Mie University Hospital; [2]Cancer Center, Mie University Hospital; [3]Central Clinical Laboratories, Mie University Hospital; [4]Transfusion Service, Mie University Hospital Mie, Japan

Abstract

Objectives: Invasive fungal diseases (IFDs) are life-threatening events in patients with haematologic disorders, and the spectrum of the aetiological pathogens continues to expand. This study aimed to evaluate the clinical utility of a panfungal polymerase chain reaction (PCR) assay for the management of IFDs in such patients. *Methods:* We prospectively analysed 273 consecutive blood samples from 64 risk episodes in 51 patients with haematologic disorders at high risk for IFD who were treated at our hospital between April 2007 and October 2010. *Results:* PCR-positive results were obtained in 18 of 64 risk episodes (35.3%). IFD was documented in 14 episodes (21.9%, 9 probable IFDs and 5 possible IFDs) according to the revised criteria of the European Organization for Research and Treatment of Cancer/Mycoses Study Group. PCR was positive in all of these 14 episodes, and in 4 of the 50 episodes with no IFD category. Sensitivity, specificity, positive predictive value, and negative predictive value of our assay were 100%, 92%, 78% and 100% respectively. A considerable number of fungi (44.4%) that are less common than *Aspergillus* and *Candida* species were positive by PCR. Molecular diagnoses of *Cunninghamella* species, *Aspergillus ustus*, *Fusarium* species, *Scedosporium apiospermum*, *Rhodotorula* species and *Rhizopus* species were beneficial in selecting suitable treatments. *Conclusions:* Our panfungal PCR approach allows for the highly sensitive and specific detection and identification of a wide spectrum of fungal pathogens, which provides indispensable information for managing IFDs, especially refractory or breakthrough IFDs during antifungal therapy in high-risk patients with haematologic disorders.

Key words polymerase chain reaction; invasive fungal disease; haematologic disorder

Correspondence Kazunori Nakase, Cancer Center, Mie University Hospital, 2-174 Edobashi, Tsu, Mie 514-8507, Japan. Tel: +81 59 231 5296, Fax: +81 59 231 5348; e-mail: k2nakase@clin.medic.mie-u.ac.jp

Accepted for publication 15 January 2013

doi:10.1111/ejh.12078

Invasive fungal diseases (IFDs) are increasingly recognised as life-threatening events in patients with haematologic disorders, particularly in patients receiving intensive chemotherapy or undergoing allogeneic stem cell transplantation (SCT) (1, 2). Early and accurate diagnosis is critical for appropriately choosing antifungal treatment, which eventually reduces the high mortality rate in these patients (3, 4). However, conventional diagnostic modalities such as radiological imaging, culture studies and histological analyses have limitations in terms of sensitivity, specificity and time taken for diagnosis (5). Moreover, serological assays for the galactomannan antigen (GM) and (1–3)-β-D-glucan (BG) cannot detect all fungal pathogens, and negative results do not completely exclude a fungal infection (6–8).

Therefore, the polymerase chain reaction (PCR) technique using fungal DNA is a promising tool for the rapid, sensitive and accurate detection and identification of causative pathogens (9, 10). However, although the spectrum of aetiological pathogens continues to expand with a growing population of immunosuppressed patients (11–13), PCR assay studies thus

far have focussed mainly on major fungi such as *Aspergillus* or *Candida* species (14–19): molecular methods for covering the diverse range of fungal genera other than *Aspergillus* or *Candida* have not been fully evaluated (19, 20).

We developed an original panfungal PCR assay system, comprising a broad-range PCR that targets the highly conserved sequence of the 18S ribosomal RNA (rRNA) gene in fungal DNA, in conjunction with genus-specific PCRs for Zygomycetes and *Fusarium*. We prospectively applied this PCR approach on fresh blood samples from patients with haematologic disorders who were at high risk for IFD, and evaluated its clinical utility for managing IFD in these patients.

Patients and methods

Patients

This study was approved by the Human Research Ethics Committee of Mie University Hospital, and written informed consent was obtained from all patients. Between April 2007 and October 2010, 51 patients (37 men, 14 women; median age, 57.5 yr; range, 17–78 yr) with haematologic disorders at high risk for IFD were enrolled in this study (Table 1). The underlying diseases included acute myeloid leukemia (AML, $n = 16$), acute lymphoblastic leukemia (ALL, $n = 5$), chronic myeloid leukaemia (CML, $n = 1$), polycythaemia vera (PV, $n = 1$), myelodysplastic syndrome (MDS, $n = 3$), Hodgkin's lymphoma (HL, $n = 1$), non-Hodgkin's lymphoma (NHL, $n = 19$), multiple myeloma (MM, $n = 4$) and aplastic anaemia (AA, $n = 1$). We analysed 64 risk episodes in these 51 patients. In accordance with the revised criteria of the European Organization for Research and Treatment of Cancer/Mycoses Study Group (EORTC/MSG) (21), a patient at high risk was defined as a patient having at least one of the following host factors: a recent history of neutropenia (<500 cells/μL), receipt of an allogeneic SCT, and prolonged use of corticosteroids (0.3 mg/kg/d of prednisone equivalent for >3 wk). Ten patients received an allogeneic SCT (three patients with AML, two with ALL, one with MDS, one with CML and three with NHL), four patients received an autologous SCT, 35 patients received chemotherapies (13 with AML, three with ALL, one with PV, one with MDS, one with HL, two with MM and 14 with NHL), and two patients received a blood transfusion only because of severe pancytopenia (one with MDS and one with AA). When the neutrophil count was <500 cells/μL, patients generally received prophylactic oral antibiotics: levofloxacin (300 mg/d) with or without sulfamethoxazole/trimethoprim (4 g/wk), and oral antifungals: fluconazole (FLCZ, 200 mg/d), itraconazole (ITCZ) oral solution (200 mg/d) or voriconazole (VCZ, 400 mg/d), and were housed in rooms with a high-efficiency particulate air filtration system. Patients with a fever (temperature >38 °C) received broad-spectrum antibiotics (β-lactams, occasionally combined with glycopeptides) as an empirical antibacterial therapy. On persistence of high fever for 3–5 d despite the use of these antibiotics, patients received empirical antifungal therapy with intravenous antifungals such as micafungin (MF, 150–300 mg/d), VCZ (400 mg/d), or liposomal amphotericin B (L-AmB, 2.5 mg/kg/d). IFD was classified as proven, probable, or possible according to the revised EORTC/MSG criteria (21).

Table 1 Patient characteristics

No. of patients	51
Median age, yr (range)	57.5 (17–78)
Sex (male/female)	37/14
Underlying disease, no. (%)	
Acute myeloid leukemia	16 (31.4)
Acute lymphoblastic leukemia	5 (9.8)
Chronic myeloid leukemia	1 (2.0)
Polycythemia vera	1 (2.0)
Myelodysplastic syndrome	3 (5.9)
Hodgkin's lymphoma	1 (2.0)
Non-Hodgkin's lymphoma	19 (37.3)
Multiple myeloma	4 (7.8)
Aplastic anemia	1 (2.0)
Treatment, no. (%)	
Chemotherapy	35 (68.6)
Allogeneic stem cell transplantation	10 (19.6)
Autologous stem cell transplantation	4 (7.8)
Blood transfusion only	2 (3.9)

Blood collection, culture, serodiagnostic tests, computed tomography of the chest and bronchoscopic examination

Ethylene-diamine-tetraacetic acid (EDTA)-anticoagulated peripheral blood samples (1 mL) were collected for PCR assay during febrile episodes, or once a week during the neutropenic period. DNA extraction and PCR analysis were performed within 24 h after collection of blood samples. For febrile episodes, blood cultures and serological GM and BG assays were performed at least once a week. Blood samples were cultured in an automated system (BacT/Alert 3D; bioMerieux, Marcy-l'Etoile, France), and the GM and BG assays were performed using the Platelia *Aspergillus* enzyme immunoassay (Bio-Rad, Marnes-la-Coquette, France), and the Beta-glucan test (Wako Pure Chemical Industries, Ltd., Osaka, Japan) respectively. GM results were recorded as an index relative to the mean optical density of the threshold controls (GM index = optical density of sample/mean optical density of the threshold control samples). A positive result was defined as an index value of ≥ 0.5. Positive BG level was defined as a serum level of ≥ 11 pg/mL. Computed tomography (CT) of the chest was performed when a patient showed a symptom of pulmonary infection or an antibiotic-resistant fever. Bronchoscopic examination with transbronchial lung biopsy (TBLB) and bronchoalveolar lavage (BAL) was performed when a pulmonary lesion was con-

firmed by a chest radiograph or CT scan, and the patient's condition permitted to do so. Cytologic analysis and GM assay were performed in BAL fluid samples.

DNA extraction

DNA from fungi was extracted and purified using a modification of previously described methods (22, 23). EDTA-anticoagulated peripheral blood was centrifuged at 1000 ***g*** for 10 min. We used the buffy coat for fungal detection. The buffy coat from whole blood was washed twice with phosphate-buffered saline and centrifuged at 3000 ***g*** for 10 min. The supernatant was decanted, and the pellet was incubated in 50 μL of lysis buffer (COBAS Amplicor S.E.T. S II kit; Roche Diagnostics, Meylan, France) at room temperature for 2 min and centrifuged at 1000 ***g*** for 1 min. The pellet was re-incubated with lysis buffer at 90 °C for 20 min and centrifuged at 13 000 ***g*** for 10 min. Fungal DNA was extracted and purified from the pellet using Mora extract (Kyokuto Seiyaku, Tokyo, Japan). Briefly, the pellet was incubated with 150 μL of lysis buffer in a tube filled with beads at 90 °C for 10 min. After vortex mixing, 200 μL of sodium dodecyl sulfate solution was added, and it was incubated at 70 °C for 10 min. Subsequently, 400 μL of phenol/chloroform mixture was added, and it was centrifuged at 13 000 ***g*** for 5 min after vortex mixing. The supernatant was transferred to a new tube, 1 mL of 99% ethanol was added and mixed and it was centrifuged at 13 000 ***g*** for 5 min. The pellet was washed with 1 mL of 70% ethanol, centrifuged at 13 000 ***g*** for 5 min and resuspended in diethylpyrocarbonate solution. To prevent contamination, all procedures were performed inside a laminar air-flow clean bench with a unidirectional workflow pattern; no more than two tubes were opened simultaneously. We used hydrophobic filter barrier pipette tips; pipettes were disinfected with 5% sodium hypochlorite and exposed to UV light after the experiments**.**

PCR analysis

The fungal 18S rRNA gene was amplified during the first PCR using primer sets: Fung-F (5′-TTCGATGGTAGGATAGTGGCC-3′) and B4R (5′-TGATCGTCTTCGATCCCCTA-3′). PCR was performed in a thermal cycler with preliminary denaturation at 94 °C for 5 min, followed by 40 amplification cycles at 94 °C for 30 s, 55 °C for 1 min and 72 °C for 1 min. All PCR-amplified products were precipitated by adding ethanol and amplified with nested PCR using primer sets: n-Fung-F (5′-GAATAAGGGTTCGATTCCGG-3′) and n-Fung-R (5′-CCCCGACCGTCCCTATTAAT-3′). Nested PCR was performed to detect small amounts of fungal DNA that could not be detected during the first PCR, and the sequence was identified. When no fungal DNA was detected by such PCR method and the clinical symptoms still continued, genus-specific PCR for Zygomycetes and *Fusarium* species were performed using specific primers for these fungi because their nucleic acids cannot be detected in the broad-range PCR. We used the following forward primers for Zygomycetes (five species): *Rhizopus* (5′-TGATCTACGTGACAAATTCT-3′), *Rhizomucor* (5′-TGATCTACGCGAGCGAACAA-3′), Mucor (5′-TGATCTACGTGACATATTCT-3′), *Absidia* (5′-TGATCTACACGGCATCAAAT-3′) and *Cunninghamella* (5′-GGATTGTAAACTAAAGTTTTC-3′), and the following reverse primers: the first four species (*Rhizopus*, *Rhizomucor*, *Mucor* and *Absidia*) (5′-AGTAGTTTGTCTTCGGKCAA-3′) and *Cunninghamella* (5′-AAATTCTCTAATTATTCCCTC-3′). For *Fusarium* species, we used the forward primer: 5′-AGTATTCTGGCGGGCATGCCTGT-3′, the first reverse primer: 5′-ACAAATTACAACTCGGGCCCGAGA-3′, and the nested reverse primer: 5′-TTGCCGCTTCACTCGCCGTTAC-3′ (24). Fungal detection sensitivity was evaluated using the test strains of *Candida albicans* (JCM1542), *Candida glabrata* (JCM1539) and *Aspergillus fumigatus* (JCM1617). As described previously (22, 23), the lower detection limit of this assay was 5×10^2 colony-forming units (CFU)/mL for the first PCR and 1×10^2 CFU/mL for the nested PCR. We validated the PCR system using various fungal strains. DNA from 20 strains of 10 bacteria and human-derived DNA were not amplified by either the first or second PCR (data not shown). The temperature conditions and number of cycles in the second PCR were the same as in the first PCR; positive and negative controls were used for each PCR assay. The positive controls were 100 CFU/mL and 500 CFU/mL *C. albicans* (JCM1542), 500 CFU/mL *C. glabrata* (JCM1539) and 500 CFU/mL *A. fumigatus* (JCM1617). The negative control was nuclease-free water. When these positive and negative controls did not work as expected, we considered the assay inappropriate. For species identification, positive PCR products were sequenced using the ABI PRISM BigDye terminator cycle sequencing ready reaction kit and ABI PRISM 377 Genetic Analyzer (Life Technologies, Tokyo, Japan). For phylogenetic identification, sequences were compared with those of known fungi listed in the official databases using the BLAST program available at the National Center for Biotechnology Information (http://ncbi.nlm.nih.gov).

Results

PCR results and its performance

In the present study, 273 PCR samples from 64 risk episodes in 51 patients were analysed, at a mean of 4.3 samples (range, 1–48) per episode. PCR positivity was observed in 18 (35.3%) of 64 episodes (Table 2). Amongst these, two or more consecutive PCR-positive results (range, 2–9) were obtained in 13 episodes, and single-positive results were found in the remaining five episodes (Table 3). In all

Table 2 Correlation of PCR results with IFD

PCR results	All episodes (n = 64)	Probable IFD	Possible IFD	No category
PCR positive	18	9	5	4
Aspergillus species	8	6	1	1
Candida species	2	1	0	1
Zygomycetes	3	1	2	0
Fusarium species	1	0	1	0
Others	4	1	1	2
PCR negative	46	0	0	46

IFD, invasive fungal disease.

episodes with multiple PCR-positive results, PCR was always positive for the same organisms. EORTC/MSG-defined IFD was documented in 14 episodes (21.9%, nine probable and five possible IFDs) (Table 2). Two episodes were included in patient 6; in one of his episodes, despite no specific findings on the chest CT scan, ocular examination showed bilateral endophthalmitis with retinal exudates; hence, this episode was classified as having possible IFD [patient 6(1)] (Table 3). PCR was positive in all of these 14 IFD episodes, and in 4 of the 50 episodes with no IFD category. Of the 61 episodes examined (17 PCR-positive and 44 PCR-negative episodes), blood cultures for fungi were negative in any case. Bronchoscopic examination was performed in three episodes (patients 2, 4 and 13). TBLB was done in one of these episodes (patient 13), and its biopsy specimen revealed the infiltration of lymphoma cells, but no focus of fungal infection. BAL fluid was obtained in the remaining two episodes, and GM was positive in one episode (patient 2), and positive cytology of myeloma cells without evidence of infection was observed in the other (patient 4). Because of no presence of proven IFD, we evaluated the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of the PCR assay for probable IFD and probable/possible IFD (Table 4). The sensitivities for probable IFD and probable/possible IFD were 100% (9/9) and 100% (14/14) respectively. The specificities for probable IFD and probable/possible IFD were 83.6% (46/55) and 92.0% (46/50) respectively. The PPVs for probable IFD and probable/possible IFD were 50.0% (9/18) and 77.8% (14/18), respectively, whereas the NPVs for probable IFD and probable/possible IFD were both 100% (46/46).

Fungal DNAs identified by PCR assay

In this study, a considerable number of fungi (44.4%) other than major ones such as *Aspergillus* and *Candida* species were positive by PCR (Tables 2 and 3). Non-major fungi identified were as follows: *Cunninghamella* species (patient 1), *Fusarium* species [patient 6(1)], *Scedosporium apiospermum* (patient 7), *Rhodotorula* species (patient 8), *Rhizopus* species (patients 10 and 11), *Paecilomyces lilacinus* (patient 15), and *Penicillium sclerotiorum* (patient 16). Major fungi identified were as follows: *A. fumigatus* [patients 2, 6(2), 12 and 14], *Aspergillus niger* (patients 3 and 9), *Aspergillus ustus* (patient 5), unknown strain of *Aspergillus* species (patient 13), *Candida parapsilosis* (patient 4) and an unknown strain of *Candida* species (patient 17).

Clinical utility of PCR results

In 10 (patients 2, 4, 5, 7, 9, 10, 12 and 15–17) of the 18 PCR-positive episodes, continued PCR screenings disclosed the clearance of the fungal DNA during antifungal therapy (Table 3). Infectious signs and/or symptoms were also improved in 9 of these 10 episodes. Although in only one episode (patient 4), this case had a ground-glass shadow on the chest CT scan and died of MM, the examination of BAL fluid before death indicated no evidence of pulmonary fungal infection. We could not take sufficient blood samples to assess the disappearance of fungal DNA in the other eight episodes, but 5 [patients 1, 6(1), 6(2), 8 and 11] of those achieved better outcomes with antifungal treatment. In the remaining three episodes, the patients (patients 3, 13 and 14) died of underlying disease; nevertheless, the autopsy findings did not confirm the focus of fungal infection. Hence, these observations seem to indicate that molecular results were useful for managing fungal infections in all of the 18 PCR-positive episodes. Amongst those, however, since neutrophil recovery was observed during the course of treatment in nine episodes which had a neutropenia when performing PCR, these situations might also contribute to the improvement of the infection.

The high value of the PCR results in selecting suitable treatments was particularly seen in the following seven cases. Because patients 1, 10 and 11 had specific pulmonary lesions without response to VCZ and had negative results for the broad-range PCR, we performed specific PCR with a strong suspicion of breakthrough zygomycosis (25, 26). Furthermore, patient 11 showed the presence of a reversed halo sign on the chest CT scan at that time, known as a radiological manifestation of zygomycosis (27). *Cunninghamella* species was detected in patient 1, and *Rhizopus* species in patients 10 and 11. Consequently, upon switching the antifungal drug to L-AmB, these patients recovered from this infection. In patient 5, although L-AmB-resistant pulmonary aspergillosis (probable IFD) persisted, identification of *A. ustus* by sequencing analysis allowed us to change the treatment regimen to the combined use of VCZ and MF (28), resulting in the improvement of this infection. Because patient 6(1) developed *Candida*-unrelated endophthalmitis during the prophylactic use of FLCZ, we performed specific PCR for *Fusarium*, an emerging pathogen of ocular disease (29). We changed the medication to VCZ (30) based on the PCR-positive result with a better out-

Table 3 Clinical characteristics of PCR positive cases

Patient no.	Age/ Sex	Underlying disease	Prior antifungal[1]	Documented fungal DNA	No. of positive (No. of assay)	Chest CT imaging	BG (pg/mL)	GM index	EORTC/MSG criteria	Treatment	Neutropenia	Outcome
1	54/F	AML	VCZ	*Cunninghamella* species	2 (2)	Nodule	Negative	Negative	Possible	L-Am B	Yes (recovery)	Improved
2	24/M	AML	ITCZ	*Aspergillus fumigatus*	9 (48)	Nodule with cavity	Negative	0.7[2]	Probable	VCZ	Yes (recovery)	Improved
3	62/M	NHL	MF	*Aspergillus niger*	2 (2)	Dense, well-circumscribed lesion	19.3	>5.0	Probable	VCZ→L-AmB[3]	Yes(recovery)	Died of NHL[4]
4	62/M	MM	FLCZ	*Candida parapsilosis*	2 (3)	Ground glass shadow	Negative	Negative	No category	VCZ	No	Died of MM[5]
5	56/M	MDS	L-AmB	*Aspergillus ustus*	4 (6)	Air-crescent sign	25.3	2.1	Probable	VCZ+MF	Yes (recovery)	Improved
6(1)[6]	75/M	NHL	FLCZ	*Fusarium* species	2 (2)	No specific finding	Negative	Negative	Possible[7]	VCZ	No	Improved
6(2)[6]	75/M	NHL	VCZ	*A. fumigatus*	2 (2)	Nodule	Negative	1.2	Probable	L-Am B	No	Improved
7	36/M	AML	ITCZ	*Scedosporium apiospermum*	2 (3)	Nodule with a halo sign	Negative	1.3	Probable	VCZ	Yes (recovery)	Improved
8	50/M	AML	MF	*Rhodotorula* species	2 (2)	No specific finding	Negative	Negative	No category	L-Am B	Yes (recovery)	Improved
9	46/M	NHL	MF	*A. niger*	4 (13)	Dense, well-circumscribed lesion	Negative	Negative	Possible	L-Am B	No	Improved
10	48/M	AML	VCZ	*Rhizopus* species	3 (13)	Small nodule	Negative	5.0	Probable	L-Am B	Yes (recovery)	Improved
11	67/M	NHL	VCZ	*Rhizopus* species	5 (5)	Nodule with a reversed halo sign	Negative	Negative	Possible	L-Am B	No	Improved
12	56/F	NHL	ITCZ	*A. fumigatus*	4 (7)	Pleural effusion	42.2	0.6	No category	VCZ	No	Improved
13	75/M	NHL	Not given	*Aspergillus* species	1 (1)	Nodule	14.4	1.3	Probable	VCZ	No	Died of NHL[4]
14	72/F	NHL	FLCZ	*A. fumigatus*	1 (1)	Nodule	Negative	0.5	Probable	L-AmB	No	Died of NHL[4]
15	44/M	MDS	Not given	*Paecilomyces lilacinus*	1 (14)	No specific finding	Negative	Negative	No category	ITCZ	Yes (recovery)	Improved
16	58/F	ALL	ITCZ	*Penicillium sclerotiorum*	1 (2)	Nodule	Negative	Negative	Possible	VCZ	No	Improved
17	20/M	ALL	FLCZ	*Candida* species	1 (2)	Nodule	11.1	Negative	Probable	VCZ	Yes (recovery)	Improved

AML, acute myeloid leukemia; NHL, non-Hodgkin lymphoma; MM, multiple myeloma; MDS, myelodysplastic syndrome; ALL, acute lymphoblastic leukemia; BG, (1–3)-β-D-glucan; GM, galactomannan; EORTC/MSG, European Organization for Research and Treatment of Cancer/Mycosis Study Group; L-AmB, liposomal amphotericin B; VCZ, voriconazole; MF, micafungin; ITCZ, itraconazole; FLCZ, fluconazole.

[1]Antifungal drug used prior to the PCR assays as a prophylaxis or empirical therapy.

[2]This result was obtained from the examination of bronchoalveolar lavage fluid.

[3]Antifungal drug was changed from VCZ to L-AmB because of VCZ induced liver injury.

[4]Autopsy did not confirm any fungal infection.

[5]Bronchoalveolar lavage before death revealed myeloma cells without evidence of infection.

[6]Two episodes were included in patient 6.

[7]Occular examination showed bilateral endophathalmitis with retinal exudates.

Table 4 Diagnostic performance of our PCR method

	Probable IFD	Probable/Possible IFD
Sensitivity	9/9 (100%)	14/14 (100%)
Specificity	46/55 (83.6%)	46/50 (92.0%)
PPV	9/18 (50.0%)	14/18 (77.8%)
NPV	46/46 (100%)	46/46 (100%)

PPV, positive predictive value; NPV, negative predictive value; IFD, invasive fungal disease.

come. Patient 7 developed a pulmonary infection during prophylaxis with ITCZ, and at that time, *S. apiospermum* was identified by PCR. VCZ treatment was effective for this patient (31). In patient 8, fever persisted irrespective of empirical MF therapy. Because PCR analysis detected *Rhodotorula* species, we changed the antifungal to L-AmB with a successful outcome (32). All of the 46 PCR-negative episodes did not result in IFD.

Comparison of PCR with GM assay in the diagnosis of aspergillosis

In this study, since no proven aspergillosis was found, we evaluated the diagnostic performance of PCR (*Aspergillus* DNA) and GM assay for detecting probable/possible IFD. Amongst six IFD episodes with positive in both tests (Table 3), PCR detection preceded GM test by 11 d in one case (patient 2). Notably, this GM detection was observed only in the BAL sample, and not in the blood samples. In the remaining five episodes, positive findings of PCR mostly synchronised with GM-positive results. The sensitivity, specificity, PPV and NPV of PCR were 50.0% (7/14), 98.0% (49/50), 87.5% (7/8) and 87.5% (49/56) respectively. Such data of GM assay were 57.1% (8/14), 90.0% (45/50), 61.5% (8/13) and 88.2% (45/51) respectively. The specificity and PPV tended to be lower in GM test than in PCR. A more likely explanation for these results is the possibility of the presence of GM false- positivity. Actually, four episodes with no IFD category showed GM-positive, but PCR-negative. In two of these episodes, GM tests seemed to be false-positive because those patients had received allogeneic SCT (33).

Discussion

In this study, we performed a panfungal PCR assay consisting of a broad-range PCR coupled with two genera-specific PCRs to detect a wide spectrum of pathogens, including emerging fungi. Because Zygomycetes and *Fusarium* species have emerged as life-threatening opportunistic pathogens and have increased in prevalence in patients with haematologic malignancies (25–27, 29), we incorporated these moulds-specific PCRs to complement the drawbacks of the broad-range PCR, in which their fungal nucleic acids cannot be detected. Subsequently, we obtained PCR-positive results in 18 of 64 episodes (35.3%) in patients with haematologic disorders at high risk for IFD. Although the present study had no proven IFD as defined by the EORTC/MSG criteria, the sensitivity and NPV of the panfungal PCR method for the 14 probable/possible IFDs were excellent; namely, both were 100%. These observations suggest that PCR-negative results may allow antifungal treatment to be withheld, and patients may be spared unnecessary therapy. However, this pre-emptive approach using a panfungal PCR assay requires evaluation in prospective randomised trials in carefully defined patient groups at high risk for IFD. The reasons for absence of proven IFD in this study are that there was only one patient in whom TBLB could be performed for the definitive diagnosis because of the patients' serious conditions (severe neutropenia and/or thrombocytopenia); moreover, none of the blood culture studies yielded any fungi, probably because most pathogens were fastidious organisms or poorly growing fungi as a result of prior antifungal use. If we had been able to perform TBLB in more cases, some episodes might be diagnosed as proven IFD. Because many patients with haematologic disorders receiving intensive chemotherapy or undergoing allogeneic SCT have similar clinical scenarios, non-invasive and repeatable PCR assay is considered to be a valuable tool for deciding the diagnosis of fungal infection.

It is noteworthy that a considerable number of fungi (8 of 18, 44.4%) other than major ones such as *Aspergillus* and *Candida* species were identified in this PCR study. Because the epidemiology of fungal infections is rapidly evolving, and non-Aspergillus, non-Candida species and other rare fungi have emerged as major opportunistic pathogens (11–13), these results apparently indicate that we should pay attention to infections caused by these uncommon fungi. Reliable serological diagnostic methods are generally absent for these organisms; hence, DNA analyses can be the only diagnostic tool when no information is obtained from the culture study. The modest PPV of the PCR assay (50.0%) for probable IFD may reflect the lack of proper serological tests for such unusual fungi for upgrading the category of possible IFD to that of probable IFD. In this study, we emphasise, in particular, that the molecular detection of Zygomycetes [*Cunninghamella* species (patient 1) and *Rhizopus* species (patients 10 and 11)], *Fusarium* species [patient 6(1)], *S. apiospermum* (patient 7) and *Rhodotorula* species (patient 8) was quite beneficial for therapeutic managements. Breakthrough zygomycoses after VCZ therapy in patients with haematologic malignancies have been increasingly recognised (26, 27). Furthermore, scedosporiosis (31), fusariosis (34, 35) and *Rhodotorula* infection (36) have been reported as emerging fatal infections in patients with haematologic disorders. Therefore, DNA-based accurate detection of such fungal pathogens might greatly influence the decisions made for these patients with respect to diagnosis. To date, however, little is known about PCR studies describing the frequent identification of uncommon fungi like our

observation. Therefore, further investigation is needed to confirm the reliability of the test in the diagnosis of such pathogens probably underestimated with conventional microbiological tests.

In regard to the major fungi, we have noted the importance of species-level identification according to the DNA sequence. Amongst these organisms, several species such as *Aspergillus terreus* (37), *A. ustus* (38, 39) and *Candida lusitaniae* (40) are known as emerging pathogens with low responsiveness to conventional antifungal drugs. Serological GM and BG assays cannot distinguish fungi to the species level, and blood culture methods are insensitive, especially in *Aspergillus* species. Therefore, confirmation of the species identity such as *A. ustus* in our study is valuable information when deciding for suitable therapeutic regimens. In addition, molecular results of common fungi such as *A. fumigatus* or *A. niger* appear to intensify the certainty of the putative diagnosis suggested by imaging and/or serological tests.

Exogenous contamination has represented a problem for interpreting results of a panfungal PCR approach like ours because some fungi are ubiquitous in the environment (19). We performed fairly strict procedures in processing PCR analysis to avoid contamination, and we believe that false positivity due to laboratory contamination is highly unlikely. However, four episodes were suspected to be false-positive because they were PCR-positives, but classified as no IFD category according to the EORTC/MSG criteria. Nevertheless, three of these (patients 4, 8 and 12) showed two or more consecutive PCR-positive results, suggesting true positivity (*C. parapsilosis*, *Rhodotorula* species and *A. fumigatus*). In their cases, a proof of infection may be obtained only by molecular analyses probably owing to the small fungal burden, which is not enough to form pulmonary lesions or is measurable only by PCR amplification because of subclinical or occult infection. Conversely, false- positivity could not be completely ruled out in patient 15 (*Paecilomyces liliacinus*), because the fungal nucleic acids were observed in only one of the 16 tests. However, the fungal DNA was cleared with therapeutic intervention, and mycosis due to this mould has been recently reported as an emerging infection in transplant recipients (41). Single PCR-positive result was also observed in four other episodes. Investigators have not adopted only one positive result as PCR positivity. In this study, three of the four episodes were classified as probable IFD (patients 13, 14 and 17), and one as possible IFD (patient 16), suggesting that these PCR-detected fungal pathogens could not be necessarily denied as causative organisms. However, the clinical significance of identifying *Penicillium sclerotiorum* in patient 16 is questionable, because, to our knowledge, no reports on infections due to this species have been found (42). In this patient, improvement of the pulmonary lesion with VCZ therapy might be attributable to the effectiveness of this agent for other undetectable fungi.

In conclusion, the panfungal PCR approach used in this study allows for the highly sensitive and specific detection and identification of a wide range of fungal pathogens, including uncommon and emerging species, even when conventional culture studies yield no causative pathogen. In addition, PCR can identify the fungal species using the sequence technique, unlike serological tests, which helps us to select a suitable antifungal therapy, especially in therapy-resistant mycosis. These traits of the panfungal PCR method appear to obviously contribute to providing indispensable information for managing IFD, particularly refractory or breakthrough IFD during prophylactic or empirical antifungal therapy in high-risk patients with haematologic disorders. However, since the main limitation of this study is the small number of patients included, large-scale studies concerning standardisation and clinical validation are necessary for these strategies to be widely accepted in the future (43).

Acknowledgements

We thank all the clinicians in the Department of Hematology and Oncology, Mie University Hospital, who assisted with the provision of data for this study. The authors declare no competing financial interests.

Funding

This work was supported in part by Grants-in-Aid from the Medical Association of Mie Prefecture in Japan.

References

1. Thaler M, Pastakia B, Shawker TH, O'Leary T, Pizzo PA. Hepatic candidiasis in cancer patients: the evolving picture of the syndrome. *Ann Intern Med* 1988;**108**:88–100.
2. Marr KA, Carter RA, Crippa F, Wald A, Corey L. Epidemiology and outcome of mold infections in hematopoietic stem cell transplant recipients. *Clin Infect Dis* 2002;**34**:909–17.
3. Aisner J, Schimpff SC, Wiernik PH. Treatment of invasive aspergillosis: relation of early diagnosis and treatment to response. *Ann Intern Med* 1977;**86**:539–43.
4. Caillot D, Casasnovas O, Bernard A, *et al.* Improved management of invasive pulmonary aspergillosis in neutropenic patients using early thoracic computerized tomographic scan and surgery. *J Clin Oncol* 1997;**15**:139–47.
5. Duthie R, Denning DW. *Aspergillus* fungemia: report of two cases and review. *Clin Infect Dis* 1995;**20**:598–605.
6. Kami M, Tanaka Y, Ogawa S, Kanda Y, Honda H, Chiba S, Mitani K, Yazaki Y, Hirai H. The limitation of the circulating *Aspergillus* antigen detection methods for BMT recipients. *Bone Marrow Transplant* 1998;**22**:832–3.
7. Marr KA, Balajee SA, Mclaughlin L, Tabouret M, Bentsen C, Walsh TJ. Detection of galactomannan antigenemia by enzyme immunoassay for the diagnosis of invasive

aspergillosis: variables that affect performance. *J Infect Dis* 2004;**190**:641–9.
8. Karageorgooulos DE, Vouloumanou EK, Ntziora F, Michalopoulos A, Rafailidis PI, Falagas ME. β-D-glucan assay for the diagnosis of invasive fungal infections: a meta analysis. *Clin Infect Dis* 2011;**52**:750–70.
9. Yamakami Y, Hashimoto A, Tokimatsu I, Nasu M. PCR detection of DNA specific for *Aspergillus* species in serum of patients with invasive aspergillosis. *J Clin Microbiol* 1996;**34**:2464–8.
10. Einsele H, Hebart H, Roller G, *et al.* Detection and identification of fungal pathogens in blood by using molecular probes. *J Clin Microbiol* 1997;**35**:1353–60.
11. Nucci M, Marr KA. Emerging fungal diseases. *Clin Infect Dis* 2005;**41**:521–6.
12. Richardson M, Lass-Flori C. Changing epidemiology of systemic fungal infections. *Clin Microbiol Infect* 2008;**14**:5–24.
13. Miceli MH, Diaz JA, Lee SA. Emerging opportunistic yeast infections. *Lancet Infect Dis* 2011;**11**:142–51.
14. Hebart H, Löffler J, Reitze H, *et al.* Prospective screening by a panfungal polymerase chain reaction assay in patients at risk for fungal infections: implications for the management of febrile neutropenia. *Br J Haematol* 2000;**111**:635–40.
15. Lass-Flörl C, Aigner J, Gunsilius E, Petzer A, Nachbaur D, Gastl G, Einsele H, Löffler J, Dierich MP, Würzner R. Screening for *Aspegillus* spp. using polymerase chain reaction of whole blood samples from patients with haematological malignancies. *Br J Haematol* 2001;**113**:180–4.
16. McLintock LA, Jones BL. Advances in the molecular and serological diagnosis of invasive fungal infection in haemato-oncology patients. *Br J Haematol* 2004;**126**:289–97.
17. Jordanides NE, Allan EK, McLintock LA, Copland M, Devaney M, Stewart K, Parker AN, Johnson PR, Holyoake TL, Jones BL. A prospective study of real-time panfungal PCR for the early diagnosis of invasive fungal infection in haemato-oncology patients. *Bone Marrow Transplant* 2005;**35**:389–95.
18. Ribeiro P, Costa F, Monteiro A, *et al.* Polymerase chain reaction screening for fungemia and/or invasive fungal infections in patients with hematologic malignancies. *Support Care Cancer* 2006;**14**:469–74.
19. Khot PD, Fredricks DN. PCR-based diagnosis of human fungal infections. *Expert Rev Anti Infect Ther* 2009;**7**:1201–21.
20. Lau A, Chen S, Sorrell T, Carter D, Malik R, Martin P, Halliday C. Development and clinical application of a panfungal PCR assay to detect and identify fungal DNA in tissue specimens. *J Clin Microbiol* 2007;**45**:380–5.
21. Pauw BD, Walsh TJ, Donnelly JP, *et al.* Revised definitions of invasive fungal diseases from the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) Consensus Group. *Clin Infect Dis* 2008;**46**:1813–21.
22. Nakamura A, Wada H, Ikejiri M, *et al.* Efficacy of procalcitonin in the early diagnosis of bacterial infection in a critical care unit. *Shock* 2009;**31**:586–91.
23. Nakamura A, Sugimoto Y, Ohishi K, *et al.* Diagnostic value of PCR analysis of bacteria and fungi from blood in empiric-therapy-resistant febrile neutropenia. *J Clin Microbiol* 2010;**48**:2030–6.
24. Hue FX, Huerre M, Rouffault MA, de Bievre C. Specific detection of fusarium species in blood and tissues by a PCR technique. *J Clin Microbiol* 1999;**37**:2434–8.
25. Imhof A, Balajee A, Fredricks DN, Englund JA, Marr KA. Breakthrough fungal infections in stem cell transplant recipients receiving voriconazole. *Clin Infect Dis* 2004;**39**:743–6.
26. Trifilio SM, Bennett CL, Yarnold PR, *et al.* Breakthrough zygomycosis after voriconazole administration among patients with hematologic malingnancies who receive hematopoietic stem-cell transplants or intensive chemotherapy. *Bone Marrow Transplant* 2007;**39**:425–9.
27. Wahba H, Truong MT, Lei X, Kontoyiannis DP, Marom EM. Reversed halo sign in invasive pulmonary fungal infections. *Clin Infect Dis* 2008;**46**:1733–7.
28. Heyn K, Tredup A, Salvenmoser S, Muller FM. Effect of voriconazole combined with micafungin against *Candida*, *Aspergillus*, and *Scedosporium* spp. and *Fusarium solani*. *Antimicrob Agents Chemother* 2005;**49**:5157–9.
29. Louie T, El Baba F, Shulman M, Jimenez-Lucho V. Endogenous endophthalmitis due to Fusarium: case report and review. *Clin Infect Dis* 1994;**18**:585–8.
30. Perfect JR, Marr KA, Walsh TJ, *et al.* Voriconazole treatment for less-common, emerging, or refractory fungal infections. *Clin Infect Dis* 2003;**36**:1122–31.
31. Husain S, Munoz P, Forrest G, Alexander BD, Somani J, Brennan K, Wagener MM, Singh N. Infections due to *Scedosporium apiospermum* and *Scedosporium profificans* in transplant recipients: clinical characteristics and impact of antifungal agent therapy on outcome. *Clin Infect Dis* 2005;**40**:89–99.
32. Diekema DJ, Petroelje B, Messer SA, Hollis RJ, Pfaller MA. Activities of available and investigational antifungal agents against *Rhodotorula* species. *J Clin Microbiol* 2005;**43**:476–8.
33. Asano-Mori Y, Kanda Y, Oshima K, *et al.* False-positive Aspergillus galactomannan antigenaemia after haematopoietic stem cell transplantation. *J Antimicrob Chemother* 2008;**61**:411–6.
34. Boutati EI, Anaissie EJ. *Fusarium*, a significant emerging pathogen in patients with hematologic malignancy: ten years' experience at a cancer center and implication for management. *Blood* 1997;**90**:999–1008.
35. Kapp M, Schargus M, Deuchert T, *et al.* Endophathalmitis as primary clinical manifestation of fatal fusariosis in an allogeneic stem cell recipient. *Transpl Infect Dis* 2011;**13**:374–9.
36. Garcia-Suarez J, Gomez-Herruz P, Cuadros JA, Burgaleta C. Epidemiology and outcome of *Rhodotorula* infection in haematological patients. *Mycoses* 2011;**54**:318–24.
37. Hachem RY, Kontoyiannis DP, Boktour MR, Afif C, Cooksley C, Bodey GP, Chatzinikolaou I, Perego C, Kantarjian HM, Raad II. *Aspergillus terreus*: an emerging Apmphotericin B-resistant opportunistic mold in patients with hematologic malignancies. *Cancer* 2004;**101**:1594–600.

38. Verweij PE, Van den Bergh MF, Rath PM, De Pauw BE, Voss A, Meis JF. Invasive aspergillosis caused by *Aspergillus ustus*: case report and review. *J Clin Microbiol* 1999;**37**:1606–9.
39. Panackal AA, Imhof A, Hanley EW, Marr KA. *Aspergillus ustus* infection among transplant recipients. *Emerg Infect Dis* 2006;**12**:403–8.
40. Atkinson BJ, Lewis RE, Kontoyiannis DP. *Candida lusitaniae* in cancer patients: risk factors for amphotericin B failure and outcome. *Med Mycol* 2008;**46**:541–6.
41. Rosmaninho A, Torres T, Velho G, Lopes V, Amorim I, Selores M. *Paecilomyces lilacinus* in transplant patients: an emerging infection. *Eur J Dermatol* 2010;**20**:643–4.
42. Szymanska J. Evaluation of mycological contamination of dental unit waterlines. *Ann Agric Environ Med* 2005;**12**:153–5.
43. Loeffler J, Barnes R, Donnelly JP. Standardization of *Aspergillus* PCR diagnosis. *Bone Marrow Transplant* 2012;**47**:299–300.

