A.Miyazato, K.Nakamura, N.Yamamoto, H.M.Mora-Montes, M.Tanaka, Y.Abe, D.Tanno, K.Inden, X.Gang, K.Ishii, K.Takeda, S.Akira, S.Saijo, Y.Iwakura, Y.Adachi, N.Ohno, K.Mitsutake, N.A.Gow, M.Kaku, K.Kawakami	Toll-like receptor 9-dependent activation of myeloid dendritic cells by deoxynucleic acids from *Candida albicans*.	Infect Immun.	77	3056-3064	2009

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Reviews

Application of *In Situ* Hybridization to Tissue Sections for Identification of Molds Causing Invasive Fungal Infection

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Abstract

The present article describes our studies to know the usefulness of *in situ* hybridization (ISH) to identify various kinds of mold observed in tissue sections and/or cytological preparations from the lesions of patients with invasive fungal infection. To establish the precise procedure for ISH in formalin-fixed and paraffin-embedded sections, various pretreatments were attempted. The condition finally chosen is written here providing a favorable outcome regarding to both intensity and specificity of signals on outline of molds observed in the tissue sections when specimens were treated with both heat and proteinase K and, solutions were adjusted to higher pH value.

Therefore, usefulness of promising probes, two each DNA and peptide nucleic acid (PNA) were verified with a favorable pretreatment condition, using lungs of mice experimentally infected and/or those obtained from autopsies with invasive mold infection. As the result, DNA probes targeting alkaline proteinase (*ALP*) gene and retrotransposon *Afut*-1 gene of *Aspergillus fumigatus* showed specific signal intensity for the *Aspergillus* species and *A. fumigatus*, respectively. PNA probes for *Candida albicans* and the *Fusarium* species also showed satisfactory specificity. We wish to emphasize that ISH can be a valuable tool to identify medically important molds in formalin-fixed and paraffin-embedded tissue sections or cytological preparations.

Key words : *in situ* hybridization, *Aspergillus fumigatus*, histopathological diagnosis, invasive fungal infection, polymerase chain reaction (PCR)

Introduction

Histopathological and cytological examination has been regarded as one of the most important diagnostic tool for invasive fungal infections. It is essentially archived by detailed observation on shape of fungi demonstrated in paraffin-embedded tissue sections or alcohol-fixed cytological specimens. Although rapid and accurate diagnosis of invasive fungal infections becomes more important for successful antifungal therapy in contemporary medicine[1, 2], it is sometime difficult to identify the fungal species in the tissue specimens, especially in case of mold infection[3 - 5]. Therefore, it is required to establish a rapid and accurate method of diagnosis in pathological fields. Whereas sensitive and rapid molecular detection assays have been recently introduced by use of polymerase chain reaction (PCR) methods to detect *Aspergillus* DNA in serum, total blood[6 - 9], or tissue samples[10, 11], it is still hard to gain satisfactory specificity. On the other hand, there have been a few attempts to use *in situ* hybridization (ISH) to identify fungi in histopathological specimens[12 - 17]. To date, the study of ISH for diag-

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Fig. 1. Flow chart of *in situ* hybridization procedure in the present study.

nosis of fungal infection with formalin-fixed and paraffin-embedded sections has not been systematically promoted. This article deals with our attempts to detect fungus-specific nucleic acids in routinely prepared tissue sections by ISH.

1. Standard procedure of ISH

In the present study, the ISH was performed with following to the procedure shown in figure 1 as the flow chart (Fig. 1).

2. Design and preparation of probes

For the present study, we employed both double stranded DNA probes and peptide nucleic acid (PNA) probes among probes previously introduced. These probes were conjugated with fluorescein isothiocyanate (FITC). After observation of FISH preparations under fluorescent microscope, FITC indicating fungi on the sections was immunohistochemically transferred to the peroxidase end product with treatment of anti-FITC antibody and horseradish peroxidase-labeled polymer solution.

Since the genes encoding virulence factors of various fungi can be used for the marker of molecular diagnosis, DNA targeting the *ALP* gene (583 bp)[12] of *A. fumigatus* was chosen for our study. In addition, *Afut-1* gene (245 bp)[18] was also chosen from retrotransposons of *A. fumigatus* for that estimated favorable specificity. For the probe-labeling assay, no agreeable outcome was gained from using nick translation technique, mostly due to fragmentation of DNA induced by the procedure (Fig. 2). Therefore, we employed either PCR fluorescein labeling mix (Rosche : 1636154) and Ulysis nucleic acid labeling kits (Molecular Probes : U-21652) which were carried out with an accordance to the manufacture's instructions. We also designed two more probes of PNA targeting the 26S rRNA of *C. albicans* and 28S rRNA of *Fusarium* species which were labeled at their N'-end with FITC (Greiner Bio-One Co. Ltd). PNA has been known as a novel type of DNA analogue[19] that has high sequence specificity and remarkable stability of both correct and mismatched PNA/DNA complexes[20-22]. In order to maximize sensitivity and specificity, we set the target of PNA probes on the rRNA derived from nuclear large subunit rDNA sequence. The sequences of nucleic acid used in the study are listed in Table 1. As a further advanced application, dual color fluorescent ISH (FISH) was carried out to visualize two different genes in a single section by adding two different probes targeting the *ALP* gene and the *Afut-1* gene to the hybridization buffer. At this time, *ALP* and *Afut-1* probes were labeled with Alexa 546 and FITC, respectively.

3. Pretreatment condition

To optimize ISH for sensitive and reproducible, a

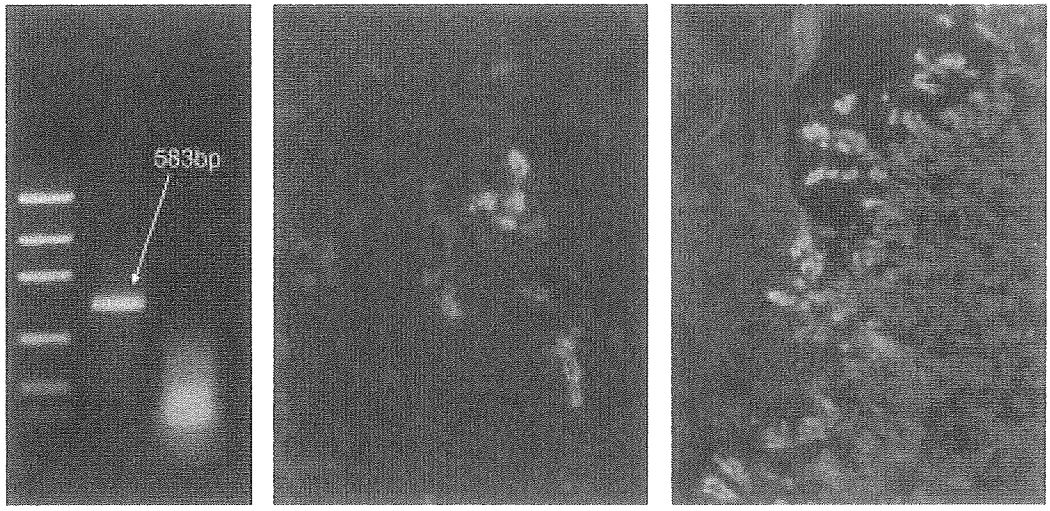


Fig. 2. Labeling of DNA by nick translation with spectrum green linked nucleotide spoiling specificity of probe, mostly due to fragmentation.

Table 1. Nucleotide sequences of the primers and probes used in the study

Probe target	Nucleotide sequence	Probe length	Probe labeling
A. fumigatus Alkaline proteinase (*ALP*)	GCCTATCCGTGTACTTGATG AAGGTTCAGCACGATCAAC J Med Microbiol **49**: 285-290, 2000[12]	583 bp ds DNA	PCR: FITC or ULYSIS®: Alexa 546
A. fumigatus Retrotransposon (*Afut-1*)	CTTTGTCACATGCTCAGAAC GGTCCTAGCCAGCGATG Nucleic Acids Res **24**: 1428-1434, 1996[18]	245bp ds DNA	PCR: FITC
Fusarium spp. 28S rRNA	GATGATCAACCAAGCCCA Shinozaki M, Nakayama H, Shibuya K: unpublished data	18 mer PNA	N': FITC
C. albicans 26S rRNA	ACAGCAGAAGCCGTG J Clin Microbiol **39**: 4138-4141, 2001[21]	15 mer PNA	N': FITC

variety of pretreatment protocol was attempted with *Afut-1* probes and sections of formalin-fixed and paraffin-embedded tissue from mice infected with *A. fumigatus* as standard materials on the basis of result from our pilot study. We firstly examined whether pH of the heat solutions may influence the quality of ISH by using five different buffer solutions: 10 mM citrates (pH 6.0 and pH 7.0), 1 mM EDTA (pH 8.0), 0.65 mM Tris-HCl EDTA (pH 9.0), and 0.1 M Tris-HCl (pH 9.5). An efficacy of proteinase K digestion with each solution was also verified, simultaneously. In general, clinical specimens for histopathological examination are routinely fixed with formalin to provide better preservation of shape and structure of cells, matrix, and tissue of the specimen, but this has been cutting two ways, because the fixation causes cross-linking of proteins and fragmentation of DNA which generally reduces the penetration of nucleic acids probes. Therefore, an appropriate unmasking of target nucleic acids and ingenious probe design must be essential to take a successful result of ISH. The heating used in the study has been adapted from antigen retrieval technique used in conventional immunohistochemistry[23]. Although the heat solutions have empirically been used for ISH on formalin-fixed and paraffin-embedded sections[13, 14], the efficiency of the detection of fungus-specific nucleic acids in formalin-fixed and paraffin-embedded sections has not been systematically verified. Shi *et al.* previously documented that the pH of heat solution was an important factor for immunostaining to archival tissue sections[24]. As the result from our trial, following procedure produced a better outcome

Table 2. Verification of various pretreatment protocols

	−	Citrate (pH 6.0) or Proteinase K	Heat-treatment※				
			Citrate (pH 6.0)	Citrate (pH 7.0)	EDTA (pH 8.0)	TE (pH 9.5)	Tris-HCl (pH 9.0)
Ethanol-fixed cytological specimens (Cultured fungi)	+++	+++					
Papanicolaou stained cytological specimens (Cultured fungi)	++	+++					
Ethanol-fixed paraffin-embedded sections (Cultured fungi)	++	+++	+++				
Formalin-fixed paraffin-embedded sections (Tissue section)	−	+	+	+～++	++	++	++

※ The tissues following heat-treatment were subjected to proteinase K (10 μg/ml) treatments for 10 min.
Degree of fluorescent intensity is indicated as follows:
−: negative, +: weak, ++: definite, +++: intense.

for ISH that is to heat tissue sections in 10 mM EDTA (pH 8.0) at 98℃ for 30 min. followed by proteinase K digestion at 37℃ for 10 min. (Table 2).

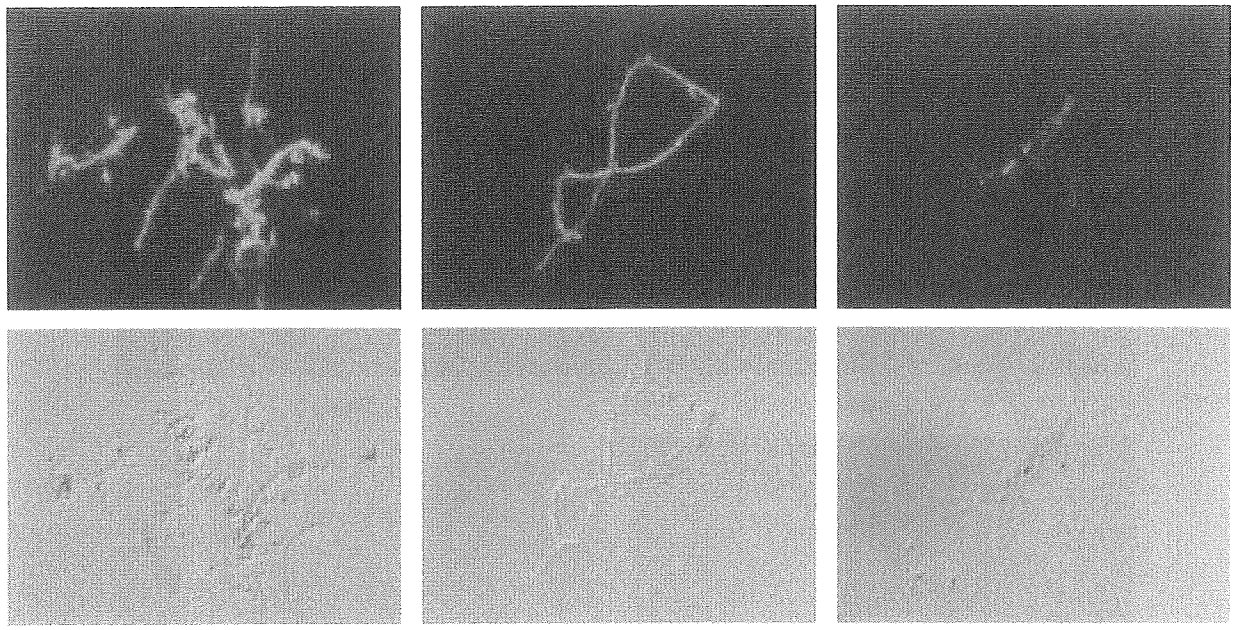
4. Specificity verification of the probes

In order to verify the specificity of probes, sections of formalin-fixed and paraffin-embedded tissues were prepared from mice experimentally infected with six different fungi comprising *A. fumigatus*, *A. terreus*, *A. niger*, *S. apiospermum*, *M. circinelloides*, and *F. solani*. As the result, the *ALP* probe reacted strongly with *A. fumigatus* and weekly with both *A. niger* and *A. terreus* (Fig. 3a). On the contrary, *F. solani*, *C. albicans*, and *R. oryzae* showed no ISH signals (Fig. 3b). The *Afut-1* probe reacted specifically with *A. fumigatus*, and there were no ISH signals in tissues infected with *A. flavus*, *A. terreus*, *F. solani*, *C. albicans*, and *R. oryzae*. The result should be significantly useful to exclude *A. terreus* from other *Aspergillus* species because *A. terreus* is less susceptible to amphotericin B *in vitro* than others[25,26]. On the other hand, both the *C. albicans* and *Fusaium* species PNA probe specifically reacted with each fungus, respectively.

5. Practice of ISH

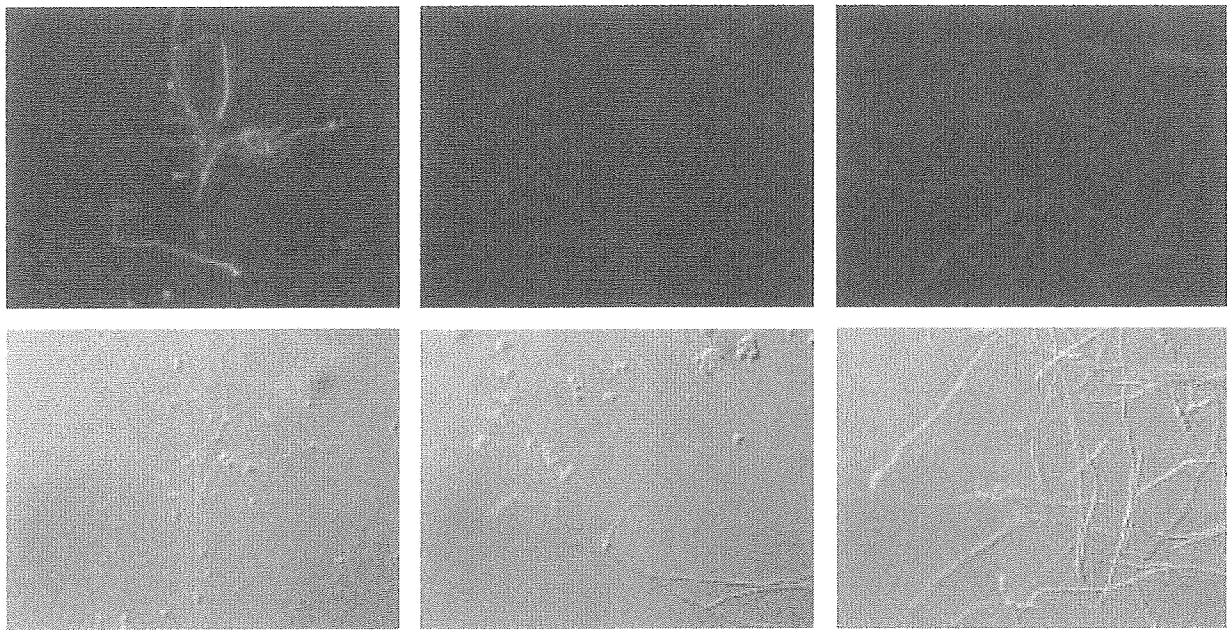
Two-color FISH method has been successfully accomplished (Fig. 4). An example of ISH is shown in Figure 5 utilizing *ALP* DNA and *C. albicans* PNA probe which was retrospectively carried out in a lesion from an autopsy case of which culture confirmed combined infection with *C. albicans* and *A. fumigatus* (Fig. 5). In addition, comparison with ISH and PCR is summarized in Table 3 using three autopsy cases *Aspergillus* infection, a case of *Zygomycetes* infection, and mice with *Fusarium* infection. We evaluated the supplemental utility of ISH conjunction with PCR for pathological diagnosis from formalin-fixed and paraffin-embedded tissue sections. Specific PCR was performed on formalin-fixed and paraffin-embedded tissue sections according to previously published techniques[8,27-29]. Fig. 6 shows the result of case #4 in Table 3. ISH showed more favorable sensitivity than PCR. Inadequately amplified PCR products surpassed at that of targeting DNA that caused false negative might explain the discrepancy. One possible reason may relate to our hypothesis that PCR-based method is affected easily by fragmentation of DNA due to formalin fixation and even if nucleic acids were suitably preserved, ISH can detect the target fungi by hybridizing to partially preserved nucleic acids. In addition, ISH may be overcome the disadvantage of PCR-based molecular techniques that has contamination risk and difficulty of DNA release in DNA extraction due to the rigid fungal cell wall.

In conclusion, ISH on formalin-fixed and paraffin-embedded tissue sections with invasive fungal infection is a valuable tool for the differentiation of medically important molds. In addition to details and careful observation on shape and distribution of mold in the



Aspergillus fumigatus *Aspergillus terreus* *Aspergillus niger*

(a)

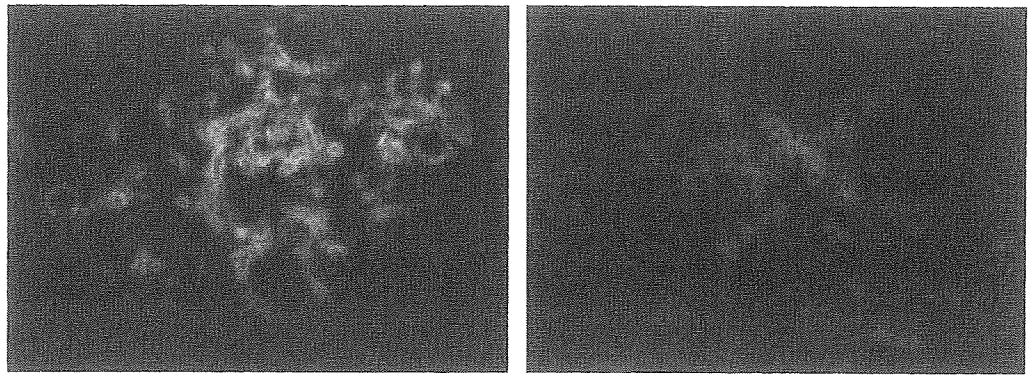


Scedosporium apiospermum *Mucor circinelloides* *Fusarium solani*

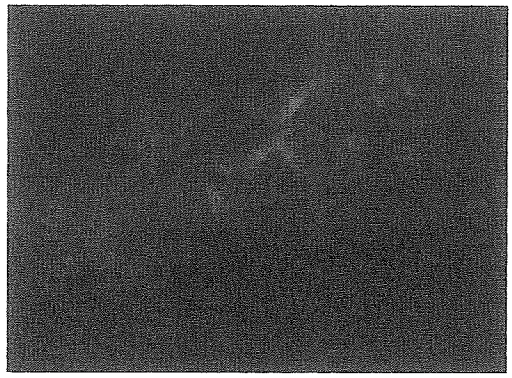
(b)

Fig. 3. Specificity verification of the probes.

Sections of formalin-fixed and paraffin-embedded tissues were employed in the study from mice infected with six different fungi comprising *A. fumigatus*, *A. terreus*, *A. niger*, *S. apiospermum*, *M. circinelloides*, and *F. solani*. As the result, the *ALP* probe reacted strongly with *A. fumigatus* and weekly with both *A. terreus*, *A. niger*, and *S. apiospermum* (a). On the contrary, *M.circinelloides* and *F. solani* showed no ISH signals (b).

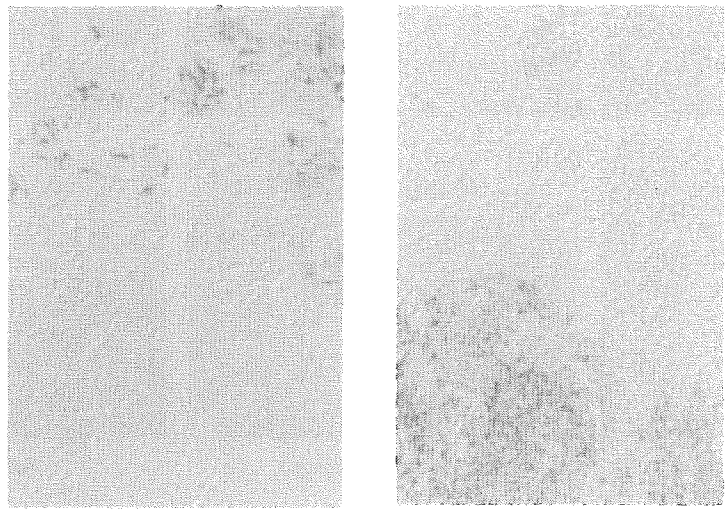


A. fumigatus *A. flavus*



A. terreus

Fig. 4. Dual color fluorescent *in situ* hybridization.
Dual color (FISH) was carried out to visualize two different genes on a single section by adding two different probes targeting the *ALP* gene and the *Afut-1* gene to the hybridization buffer. At this photograph, *ALP* and *Afut-1* probes were labeled with Alexa 546 and FITC, respectively.



The PCR probe targeting the *ALP* gene of *A. fumigatus*

The PNA probe targeting the 26S rRNA *of C. albicans*

Fig. 5. An example of ISH utilizing *ALP* DNA and *C. albicans* PNA probe showing clear identification between *C. albicans* and *A. fumigatus* on the section of combined infection from an autopsy.

Table 3. Comparison between ISH and PCR

Case	Age/sex	Organs	Underlying disease	Diagnosis	*Afut-1*	*ALP*	ISH ALPns ALPnr	ISH LTR1n LTR2n	PCR AFU5S AFU5AS	PCR P58SL P28SL	PCR ZM1 ZM3	PCR Pbo1 SP2
1	57/M	Brain	AML (M2)	Zygomycosis	−	−	−	−	−	−	+	−
2	54/M	Lung	AML (M2)	IPA	+	+	−	−	−	−	−	−
3	37/F	Lung	CML	IPA	+	+	−	−	+	−	−	−
4	57/F	Lung	NHL	IPA	+	+	−	+	+	−	−	−
5	Experimental invasive fusariosis Lung				−	−	−	−	−	+	−	−
6	69/M	Lung	AML (M5)	IPA	/	+	/	/	+	/	−	/
				Zygomycosis	/	−	/	/	−	/	+	/

IPA: Invasive Pulmonary Aspergillosis, AML: Acute Myeloid Leukemia,
CML: Chronic Myelogenous Leukemia, NHL: Non Hodgkin's Lymphoma,

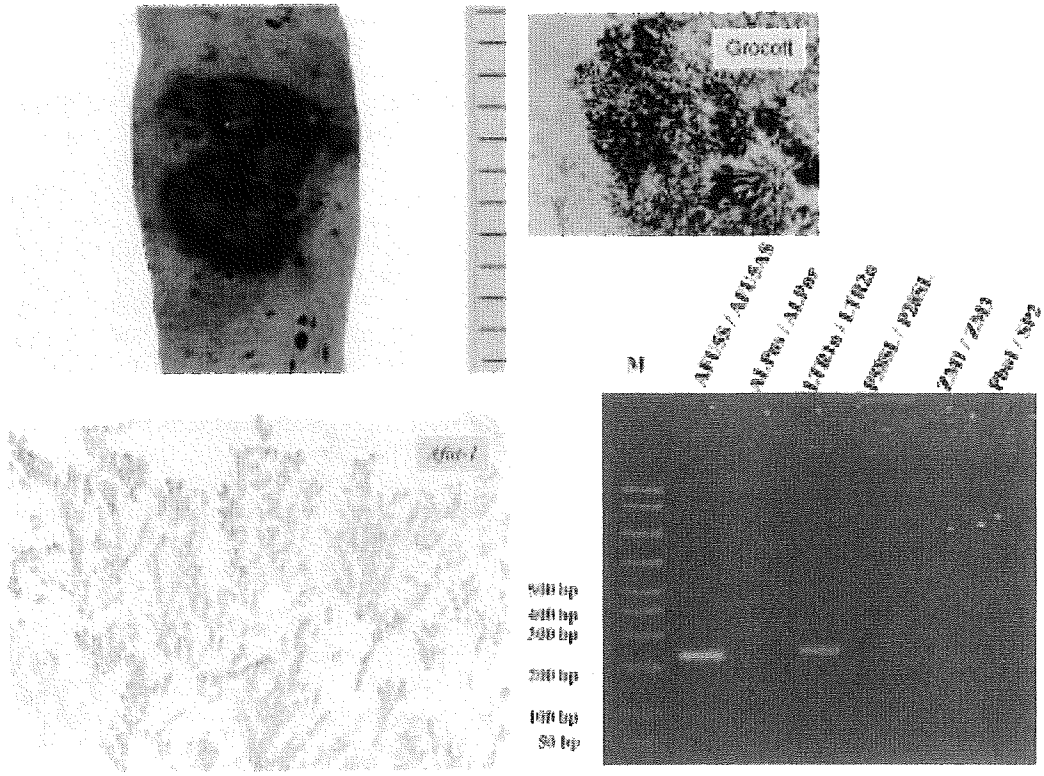


Fig. 6. Case #4 in Table 3. ISH shows more favorable sensitivity than PCR. Section of the lung, microphotograph of section stained with Grocott's, result of ISH with *Afut-1*, and PCR are shown from the left upper to the right lower corner.

tissue sections, ISH targeting specific DNA and rRNA of mold must provide an significant contribution to rapid and accurate diagnosis of the disease. An additional goal of our study should be to design probes against *Scedosporium* species and Zygomycetes.

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References

1) Anaissie EJ, Bodey GP, Rinaldi MG: Emerging fungal pathogens. Eur J Clin Microbiol Infect Dis **8**: 323-330, 1989.
2) McNeil MM, Nash SL, Hajjeh RA, Phelan MA, Conn LA, Plikaytis BD, Warnock DW: Trends in mortality due to invasive mycotic diseases in the United States, 1980-1997. Clin Infect Dis **33**: 641-647, 2001.
3) Shibuya K, Paris S, Ando T, Nakayama H, Hatori T, Latge JP: Catalases of *Aspergillus fumigatus* and inflammation in aspergillosis. Jpn J Med Mycol **47**: 249-255, 2006.
4) Lin SJ, Schranz J, Teutsh SM: Aspergillosis case-fatality rate: systematic review of the literature. Clin Infect Dis **32**: 358-366, 2001.
5) Shibuya K, Ando T, Hasegawa C, Wakayama M, Hamatani S, Hatori T, Nagayama T, Nonaka H: Pathophysiology of pulmonary aspergillosis. J Infect Chemother **10**: 138-145, 2004.
6) Makimura K, Murayama SY, Yamaguchi H: Detection of a wide range of medically important fungi by the polymerase chain reaction. J Med Microbiol **40**: 358-364, 1994.
7) Klingspor L, Jalal S: Molecular detection and identification of *Candida* and *Aspergillus* spp. from clinical samples using real-time PCR. Clin Microbiol Infect **12**: 745-753, 2006.
8) Skladny H, Buchheidt D, Baust C, Krieg-Schneider F, Seifarth W, Leib-Mösch C, Hehlmann R: Specific detection of *Aspergillus* species in blood and bronchoalveolar lavage samples of immunocompromised patients by two-step PCR. J Clin Microbiol **37**: 3865-3871, 1999.
9) Einsele H, Hebart H, Roller G, Löffler J, Rothenhofer I, Müller CA, Bowden RA, van Burik J, Engelhard D, Kanz L, Schumacher U: Detection and identification of fungal pathogens in blood by using molecular probes. J Clin Microbiol **35**: 1353-1360, 1997.
10) 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 **45**: 380-385, 2007.
11) Sandhu GS, Kline BC, Stockman L, Roberts G D: Molecular probes for diagnosis of fungal infections. J Clin Microbiol **33**: 2913-2919, 1995.
12) Hanazawa R, Murayama SY, Yamaguchi H: *In-situ* detection of *Aspergillus fumigatus*. J Med Microbiol **49**: 285-290, 2000.
13) Hayden RT, Qian X, Roberts GD, Lloyd RV: *In situ* hybridization for the identification of yeastlike organisms in tissue section. Diagn Mol Pathol **10**: 15-23, 2001.
14) Hayden RT, Qian X, Procop GW, Roberts GD, Lloyd RV: *In situ* hybridization for the identification of filamentous fungi in tissue section. Diagn Mol Pathol **11**: 119-126, 2002.
15) Montone KT, Litzky LA: Rapid method for detection of *Aspergillus* 5S ribosomal RNA using a genus-specific oligonucleotide probe. Am J Clin Pathol **103**: 48-51, 1995.
16) Kobayashi M, Sonobe H, Ikezoe T, Hakoda E, Ohtsuki Y, Taguchi H: *In situ* detection of *Aspergillus* 18S ribosomal RNA in invasive pulmonary aspergillosis. Intern Med **38**: 563-569, 1999.
17) Zimmerman RL, Montone KT, Fogt F, Norris AH: Ultra fast identification of *Aspergillus* species in pulmonary cytology specimens by *in situ* hybridization. Int J Mol Med **5**: 427-429, 2000.
18) Neuveglise C, Sarfati J, Latge JP, Paris S: *Afut1*, a retrotransposon-like element from *Aspergillus fumigatus*. Nucleic Acids Res **24**: 1428-1434, 1996.
19) Nielsen PE, Egholm M, Berg RH, Buchardt O: Sequence-selective recognition of DNA by strand displacement with a thymine-substituted polyamide. Science **254**: 1497-1500, 1991.
20) Wilson DA, Joyce MJ, Hall LS, Reller LB, Roberts GD, Hall GS, Alexander BD, Procop GW: Multicenter evaluation of a *Candida albicans* peptide nucleic acid fluorescent *in situ* hybridization probe for characterization of yeast isolates from blood cultures. J Clin Microbiol **43**: 2909-2912, 2005.
21) Oliveira K, Haase G, Kurtzman C, Hyldig-Nielsen JJ, Stender H: Differentiation of *Candida albicans* and *Candida dubliniensis* by fluorescent *in situ* hybridization with peptide nucleic acid probes. J Clin Microbiol **39**: 4138-4141, 2001.
22) Demidov VV, Yavnilovich MV, Belotserkovskii BP, Frank-Kamenetskii MD, Nielsen PE: Kinetics and mechanism of polyamide ("peptide") nucleic acid binding to duplex DNA. Proc Natl Acad Sci USA **92**: 2637-2641, 1995.
23) Shi SR, Key ME, Kalra KL: Antigen retrieval in formalin-fixed, paraffin-embedded tissues: an enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections. J Histochem Cytochem **39**: 741-748, 1991.
24) Shi SR, Imam SA, Young L, Cote RJ, Taylor CR: Antigen retrieval immunohistochemistry under the influence of pH using monoclonal antibodies. J Histochem

Cytochem **43**: 193-201, 1995.
25) Lass-Flörl C, Kofler G, Kropshofer G, Hermans J, Kreczy A, Dierich MP, Niederwieser D: *In-vitro* testing of susceptibility to amphotericin B is a reliable predictor of clinical outcome in invasive aspergillosis. J Antimicrob Chemother **42**: 497-502, 1998.
26) Sutton DA, Sanche SE, Revankar SG, Fothergill AW, Rinaldi MG: *In vitro* amphotericin B resistance in clinical isolates of *Aspergillus terreus*, with a head-to-head comparison to voriconazole. J Clin Microbiol **37**: 2343-2345, 1999.
27) 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 **37**: 2434-2438, 1999.
28) Bialek R, Konrad F, Kern J, Aepinus C, Cecenas L, Gonzalez GM, Just-Nübling G, Willinger B, Presterl E, Lass-Flörl C, Rickerts V: PCR based identification and discrimination of agents of mucormycosis and aspergillosis in paraffin wax embedded tissue. J Clin Pathol **58**: 1180-1184, 2005.
29) Hagari Y, Ishioka S, Ohyama F, Mihara M: Cutaneous infection showing sporotrichoid spread caused by *Pseudallescheria boydii* (*Scedosporium apiospermum*): successful detection of fungal DNA in formalin-fixed, paraffin-embedded sections by seminested PCR. Arch Dermatol **138**: 271-272, 2002.

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Original Article

Pathophysiological Study of Chronic Necrotizing Pulmonary Aspergillosis

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SUMMARY: The aim of the present study is to define the characteristics of the clinical and histopathological features of chronic necrotizing pulmonary aspergillosis (CNPA) cases with severe hemoptysis. We conducted a histological study of three patients clinically diagnosed as having CNPA who had hemoptysis for 5 years. A tuberculosis sequelae was found as the underlying disorder in all three cases. All patients had fever, general fatigue, and hemoptysis, and their chest computed tomographic images revealed fungus balls, cavity wall thickening, consolidation surrounding the cavity, and satellite foci. All had been treated with anti-fungal drugs and corticosteroids. However, all patients died from respiratory failure due to massive hemoptysis. Histopathological examination revealed that the cavity wall consisted of three layers comprised of necrotic, granulation, and fibrous tissue layers. *Aspergilli* were found in both the fungus ball and necrotic tissue comprising the inner layer of the cavity. In addition, most of the vessels were incompletely occluded with thrombosis and were necrotic, as well as showing local invasion of *Aspergilli*. Surgical intervention should be considered as a prior procedure for CNPA patients, because vessels at the cavity wall, whether occluded completely or incompletely, are usually necrotic and/or show local invasion of *Aspergilli*.

INTRODUCTION

Binder et al. (1) defined the term chronic necrotizing pulmonary aspergillosis (CNPA) to describe the state between aspergilloma and invasive pulmonary aspergillus. Although several recent reports have led to a better understanding of the clinical features of CNPA (2), its pathological features and mechanisms of hemoptysis still remain obscure. To obtain a detailed understanding of the pathophysiology of CNPA and hemoptysis as its serious complication, both the clinical courses and histological alterations observed in three autopsies of CNPA were examined. These three cases had been transferred from non-invasive aspergillosis of fungal ball type occurred in sequelae of pulmonary tuberculosis.

PATIENTS AND LABORATORY INFORMATION

Case 1: A 67-year-old man with sequelae of pulmonary tuberculosis and hepatitis C virus (HCV)-related liver cirrhosis was admitted to our hospital in October 2001 with a 4-month history of cough, hemoptysis, wheezing, and dyspnea. Physical examination revealed a body temperature of 37.4°C, blood pressure of 134/86 mmHg, and pulse rate of 68/min with an irregular rhythm. The auscultation indicated wheezing in the bilateral lung fields. The laboratory data revealed a leukocyte count of 3,800/μl with 62.5% neutrophils, a platelet count of 9.4 × 10^3/μl, and PaO_2 of 64 torr and $PaCO_2$ of 62 torr on room air. Serum precipitation antibody for *Aspergillus* was positive, and β-D-glucan was elevated at 247 pg/ml. *Aspergillus* galactomannan was not examined. The chest X-ray showed pleural thickening in the right upper lung field (Fig. 1a). Chest computed tomography (CT) scan revealed a mycetoma of 20 × 18 mm in size within the thick-walled cavity which was encompassed with consolidation (Fig. 1b). No mycetomas were isolated from the sputum and no fungal, bacterial, and mycobacterial pathogens were found. He was started with a single 200-mg dose of oral itraconazole (ITCZ) and 40-mg dose of prednisolone for complications of bronchial asthma. On the 20th day after the initiation of the treatment, chest X-ray revealed progressive ground glass opacities in the bilateral lower lung fields. He was administered a high dose of intravenous corticosteroid under the diagnosis of suspected drug-induced ITCZ-induced interstitial pneumonia. After receiving intravenous corticosteroid pulse therapy, the clinical symptoms and chest CT images temporarily improved. However, on the 80th day, the patient died of respiratory failure caused by massive hemoptysis.

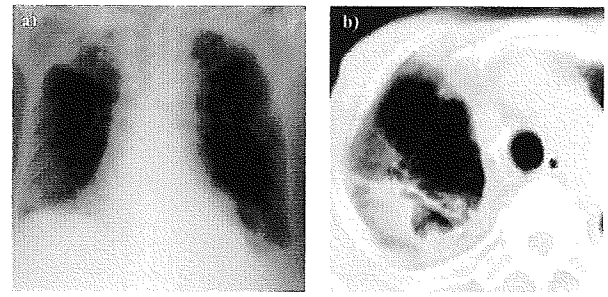

Fig. 1. (a) Chest radiograph on admission showed pleural thickening in the right upper lung field (Case 1). (b) Chest CT showed a mycetoma of 20×18 mm in size within the cavity, the wall of which was thickened and had invaded the surrounding parenchyma (Case 1).

Case 2: A 77-year-old man with sequelae of pulmonary tuberculosis was admitted to our hospital in March 2000 with a 4-month history of fever, hemoptysis, and dyspnea. He had been treated with oral antibiotics beginning 3 months before admission. The chest X-ray showed a cavity in the left upper lung field. Physical examination revealed a body temperature of 38.2°C, blood pressure of 110/64 mmHg, and pulse rate of 96/min with regular rhythm. The auscultation indi-

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cated coarse crackles in the bilateral lung fields. Laboratory data revealed a leukocyte count of 7,200/μl with 82.5% neutrophils, PaO_2 of 64 torr and $PaCO_2$ of 62 torr on 50% oxygen mask. Sputum culture isolated *Mycobacterium avium* complex. The serum precipitation antibody for *Aspergillus* was positive, and β-D-glucan and galactomannan were elevated at 21.8 pg/ml and 2.5 ng/ml, respectively. Chest X-ray showed a cavity with thickening of the adjacent pleura in the left upper lung field and consolidation in the right upper and middle lung fields (Fig. 2a). Chest CT scan revealed a mycetoma of 28 × 18 mm in size within the cavity, and the consolidation was confirmed with air bronchograms (Figs. 2b, 2c). He was started with a single 10-mg dose of intravenous AMPH-B (amphotericin-B deoxycholate 0.25 mg/kg body weight), 200-mg of oral ITCZ, 200-mg of oral sparfloxacin, and 800-mg of oral erythromycin. After the initiation of these treatments, the clinical symptoms improved and the chest CT showed significant improvement of consolidation and a decrease in the size of the mycetoma. Just before death, no acid-fast bacilli were found in the sputum. However, on the 44th day after treatment, he died of refractory respiratory failure caused by the sudden onset of massive hemoptysis.

Case 3: A 77-year-old woman with sequelae of pulmonary tuberculosis and HCV-related liver cirrhosis was admitted to our hospital in March 2004 with a 1-month history of fever,

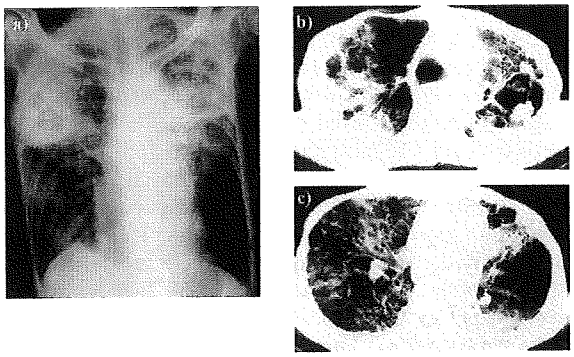

Fig. 2. (a) Chest radiograph revealed a cavitary lesion in the left upper lung field and infiltrative shadow in the right upper and middle lung fields (Case 2). (b), (c) Chest CT showed a fungus ball in the cavity in the left upper lobe and consolidation in the right upper and middle lobes (Case 2).

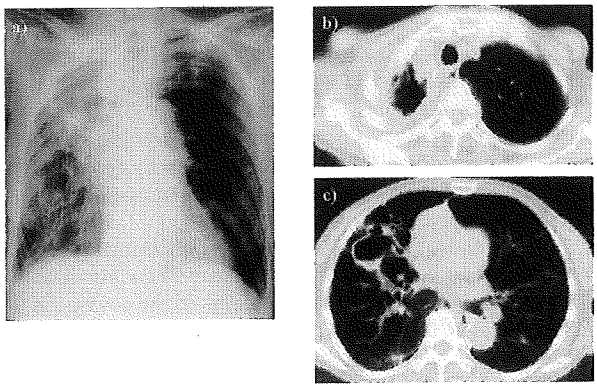

Fig. 3. (a) Chest radiograph showing a cavitary lesion in the right upper lung field and infiltrative shadow surrounding the cavitation (Case 3). (b), (c) Chest CT scan showing a cavity with adjacent pleural thickening in the right upper lobe and cavitary infiltrates with fluid collection in the middle lobe (Case 3).

hemoptysis, and dyspnea. She had a temperature of 37°C, blood pressure of 134/70 mmHg, and pulse rate of 90/min with a regular rhythm. The chest auscultation revealed coarse crackles in the bilateral lung fields. Laboratory data revealed a leukocyte count of 11,500/μl with 70.5% neutrophils, a platelet count of 9.4 × $10^3/\mu$l, and PaO_2 of 54 torr and $PaCO_2$ of 42 torr on room air. Culture of sputum was negative for fungal, bacterial, or mycobacterial pathogens. Serum precipitation antibody for *Aspergillus* was positive and β-D-glucan was elevated at 27.4 pg/ml, but galactomannan was not elevated (0.2 ng/ml). Chest X-ray showed a cavity with adjacent pleural thickening in the right upper lung field and consolidation in the right upper and middle lung fields (Fig. 3a). Chest CT scan revealed a cavity of 70 × 55 mm in size and the consolidation was confirmed (Figs. 3b, 3c). She had been treated with a single 150-mg dose of micafungin and 20-mg of prednisolone for the organizing pneumonia. After the initiation of antifungal treatment, the clinical symptoms and chest CT findings were improved. However, on the 37th day the patient died of respiratory failure resulting from massive hemoptysis.

RESULTS

Case 1: Macroscopic examination of the right upper lobe revealed a cavity of 50 mm in diameter, which was filled with a fungus ball and coagulated exudate (Fig. 4a). Histological examination revealed that the cavity wall was composed of three concentric circular layers: necrotic, granulation, and fibrous tissue layers arranged from the luminal position (Fig. 4b). Numerous hyphae were present in the fungus ball and necrotic tissue, and some had invaded into blood vessels (Fig. 4c).

Case 2: Macroscopically, a cavity measuring 50×40 mm in size was found at the section of the left upper lobe which was filled with coagulated bloody exudate corresponding to a mycetoma (Fig. 5a). Numerous hyphae were confirmed by histological examination in the coagulation of exudate (Fig. 5b). However, a few hyphae were seen as invading organisms into the blood vessels wall (Fig. 5c).

Case 3: Macroscopic examination showed a thin-walled

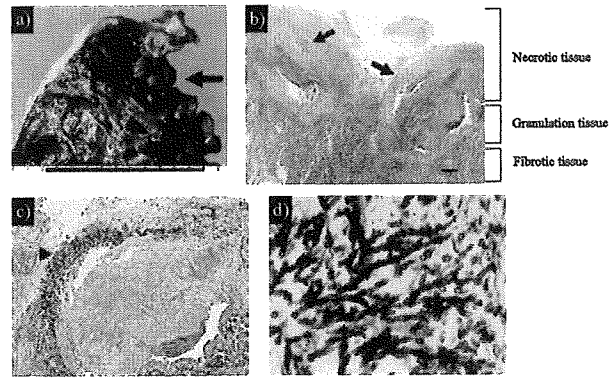

Fig. 4. (a) Macroscopic appearance of the right upper lobe demonstrated a cavity 50 mm in diameter, which was filled with a fungus ball and necrotic materials (arrow) (Case 1). Scale: 1 division = 0.5 cm. (b) Histopathological appearance of the cavity wall revealed three layers, which were of necrotic, granulation and fibrotic tissue (arrows) (Hematoxylin-Eosin stain). Scale bar = 250 μm. (c) Many fungi had invaded the blood vessel (arrow head) (Grocott's stain). Scale bar = 200 μm. (d) There were numerous *Aspergillus* hyphae with Y-shaped branching.

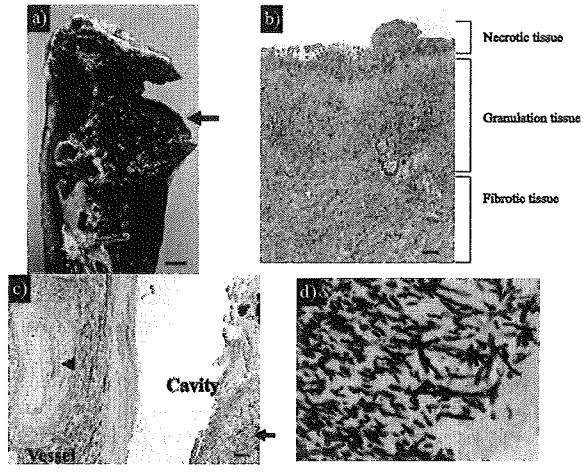

Fig. 5. (a) Macroscopic examination of the left upper lobe revealed a cavity 5 cm in diameter, which was filled with a fungus ball and necrotic materials (arrow) (Case 2). Scale bar = 1 cm. (b) Histopathology of the cavity wall revealed three layers: necrotic, granulation, and fibrotic tissue (Hematoxylin-Eosin stain). Scale bar = 150 μm. (c) Many fungi were evident in the fungus ball and in the cavity (arrow), and had invaded into blood vessel (arrow head) (Grocott's stain). Scale bar = 200 μm. (d) There were densely intertwined separated hyphae consistent with *Aspergillus* spp.

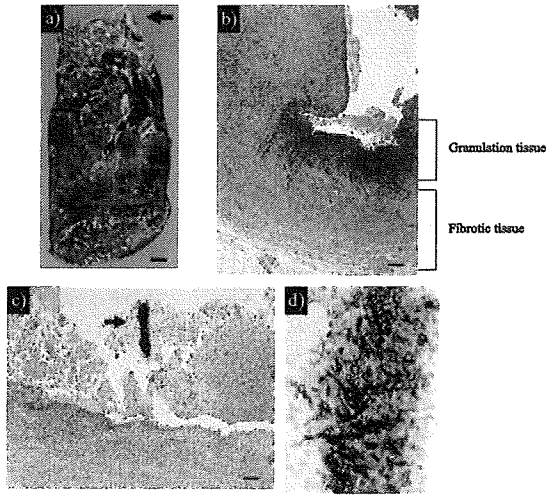

Fig. 6. (a) Macroscopic examination of the right upper lobe revealed a cavity wall 70 mm in diameter (arrow) (Case 3). Scale bar = 1 cm. (b) Microscopic examination of the cavity wall revealed two layers, which were granulation tissue and fibrotic tissue, and the absence of necrotic tissue (Hematoxylin-Eosin stain). Scale bar = 300 μm. (c) Some fungi were noted in the fungus ball (arrow) (Grocott's stain). Scale bar = 170 μm. (d) Fungal elements, showing the branching separated *Aspergillus* hyphae.

cavity of 70 mm in diameter on the section of the right upper lobe (Fig. 6a). Histologically, the cavity wall was comprised of thin and dense fibrous tissue covered with a little necrotic material (Fig. 6b). No hyphae were found as invading organisms in the cavity wall. Hyphae of fungi were limited in the intracavitary mycetoma in the case (Fig. 6c). No fungi were also found in the organizing lesions corresponding to the area of consolidation around the cavity on the chest X-ray.

DISCUSSION

Binder et al. (1) proposed that CNPA is a disease pattern that is independent of the pulmonary aspergillosis that characteristically develops in patients with mild immunosuppression, including those with diabetes mellitus, malnutrition, long-term use of low-dose corticosteroids, and collagen vascular diseases such as ankylosing spondylitis and rheumatoid arthritis. Although the clinical course is a chronic process that progresses slowly over several months to years, cavitations occur continuously due to hyphae invading into the tissues, and there is no vascular invasion or dissemination to other organs. This was followed by Gefter et al. (3), who defined semi-invasive aspergillosis as a lesion accompanied by destruction of the lung without fungus invasion of the tissues. As previously mentioned, this form may be accepted as the state during which transformation from the non-invasive form to invasive pulmonary disease occurs.

CNPA usually occurs in middle-aged and elderly patients with underlying lung diseases inducing anatomical remodeling of peripheral airways, such as in chronic obstructive pulmonary disease (COPD), old tuberculosis, pneumoconiosis, cystic fibrosis, and sarcoidosis, and is more likely to develop if the previous anatomical alteration has progressed, such as with inactive tuberculosis with residual cavities (4). The patient usually has fever, cough, sputum, and weight loss of 1 to 6 months duration (4). Radiographic findings show progressive upper lobe cavitary infiltrates associated with pleural thickening. Mycetomas are seen in about half of the patients (5). In the present study, the mean age of the three patients examined was 73.7 ± 8.8 years old. The underlying pulmonary disorders were sequelae of pulmonary tuberculosis in all patients, and two had HCV-related liver cirrhosis as the systemic underlying disease. All presented with chronic cough, hemoptysis, fever, and dyspnea. Chest radiograph revealed a cavity filled with a fungus ball at the upper lobes as a common finding in all patients. Although various histopathological alterations of CNPA have been reported, no histopathological definition has been accepted for diagnosis (2). Therefore, in the present study, the three patients with CNPA were clinically diagnosed in accordance with criteria proposed by Kohno et al. (6). The criteria are: (i) chronic symptoms with fever, cough, hemoptysis, and body weight loss, (ii) chest X-ray and CT scan abnormalities showing infiltrates and cavities in the upper lobes, (iii) positive levels of serum precipitation antibody for *Aspergillus* and β-D-glucan; and/or, (iv) isolation of *Aspergillus* spp. from lung specimens, and (v) failure to detect other bacterial, fungal, or mycobacterial pathogens. According to these criteria, the three patients were diagnosed as having CNPA.

Our pathological examination confirmed fungus balls, including necrotic material within the cavity in which numerous fungi were present. There was, however, an absence of dissemination to other organs observed by autopsy examination. In addition, the lungs in all present cases showed no histopathological findings of bronchopneumonia or nontuberculosis mycobacterial pulmonary disease.

Consequently, this suggested that the organization around the cavity may be caused by degenerated exudate disseminated and provided from the mycetoma in the cavity via the airway. The presence of an organizing lesion without fungal components around the cavity is a significantly different characteristic from that of invasive pulmonary aspergillosis (IPA), because the infiltrative shadow in the case of IPA essentially mirrors the filling of acute inflammatory exudates in the alveoli with a fungal proliferation (7,8). Protease and other considerable cytotoxic agents produced by *Aspergilli* (9,10)

may also play a role in producing and increasing the organizing lesion in CNPA. It has been known that long-term corticosteroid administration, diabetes mellitus, and liver cirrhosis impair the function of neutrophils, which play an important role in preventing the invasion of the hyphae of *Aspergilli* from the cavity to the wall covered with epithelium (11).

All of the present patients died of respiratory failure caused by massive hemoptysis. It has been known that massive hemoptysis in the case of IPA is caused by destruction of vessels by direct invasion of *Aspergilli*. However, hemoptysis, understood as bleeding of the peripheral airway, in CNPA may result from invasion of *Aspergilli* in the vessels walls which is limited in necrotic layer of the cavity wall and that lumen is largely occluded by thrombi. Hebisawa et al. (12) reported that hemoptysis occurred in patients with CNPA caused by rupturing of the pulmonary artery, which had been exposed to the luminal surface of the cavity. They assumed that fibrinoid necrosis may weaken the pulmonary arterial wall and that high blood pressure from the bronchial and intercostal arteries may cause the pulmonary artery to rupture. From our observation, an exposure of blood vessels was confirmed, but most of them were occluded incompletely by thrombosis. Therefore, destruction of a plural number of vessels involved by necrosis, local invasion of *Aspergilli*, and the break down of necrotic tissue itself into the cavity may cause bleeding. Moreover, coagulation abnormalities due to liver cirrhosis revealed by the patients of Cases 1 and 3 might be the trigger for hemoptysis.

We surmised that although the clinical signs and data indicate that fungal growth is restrained by antifungal therapy, the patient still is at continuous risk for sudden onset of massive hemoptysis, because the blood vessels, even if they are mostly occluded, may be exposed to the lumen and progressively degenerated by involvement of necrosis at the cavity wall. A coagulation abnormality is commonly associated with such patients. Thus, pulmonary resection is one of the agreeable procedure to improve the outcome for patients with IPA with neutropenia (13,14).

In conclusion, surgical intervention should be considered as a prior procedure for the disease, even though the invasion of *Aspergilli* is essentially limited to the eroded and necrotic area of the cavity wall in CNPA, because vessels at the cavity wall, whether occluded completely or incompletely, are usually involved by necrosis and/or local invasion of *Aspergilli*, as confirmed by the present study.

REFERENCES

1. Binder, R.E., Faling, L.J., Pugatch, R.D., et al. (1982): Chronic necrotizing pulmonary aspergillosis: a discrete clinical entity. Medicine, 61, 109-124.
2. Yousem, S.A. (1997): The histological spectrum of chronic necotizing forms of pulmonary aspergillosis. Hum. Pathol., 28, 650-656.
3. Gefter, W.B., Weingrad, T.R., Epstein, D.M., et al. (1981): Semi-invasive pulmonary aspergillus. Radiology, 140, 313-321.
4. Soubani, A.O. and Chandrasekar, P.H. (2002): The clinical spectrum of pulmonary aspergillosis. Chest, 121, 1988-1999.
5. Saraceno, J.L., Phelps, D.T., Ferro, T.J., et al. (1997): Chronic necrotizing pulmonary aspergillosis: approach to management. Chest, 112, 541-548.
6. Kohno, S., Masaoka, T., Yamaguchi, H., et al. (2004): A multicenter, open-label clinical study of micafungin (FK463) in the treatment of deep-seated mycosis in Japan. Scand. J. Infect. Dis., 36, 372-379.
7. Shibuya, K., Ando, T., Wakayama, M., et al. (1997): Pathological spectrum of invasive pulmonary aspergillosis. Jpn. J. Med. Mycol., 38, 175-181 (in Japanese).
8. Sugino, K., Hasegawa, T., Kimura, K., et al. (2007): Clinicopathological study of invasive pulmonary aspergillosis complicated by leukemia. J. Jpn. Assoc. Infect. Dis., 81, 261-267 (in Japanese).
9. Amitani, R., Taylor, G., Elezis, E.N., et al. (1995): Purification and characterization of factors produced by aspergillus fumigatus which affect human clinical respiratory epithelium. Infect. Immun., 63, 3266-3271.
10. Murayama, T., Amitani, R., Ikegami, Y., et al. (1996): Suppressive effects of aspergillus fumigatus culture filtrates on human alveolar macrophages and polymorphonuclear leucocytes. Eur. Respir. J., 9, 293-300.
11. Shibuya, K., Ando, T., Hasegawa, C., et al. (2004): Pathophysiology of pulmonary aspergillosis. J. Infect. Chemother., 10, 138-145.
12. Hebisawa, A., Tamura, A., Baba, M., et al. (2003): Histopathology of saprophytic aspergillosis, including hemoptysis. Jpn. J. Chest Dis., 62, 1070-1079 (in Japanese).
13. Reichenberger, F., Habicht, J., Kaim, A., et al. (1998): Lung resection for invasive pulmonary aspergillosis in neutropenic patients with hematologic diseases. Am. J. Respir. Crit. Care Med., 158, 885-890.
14. Pidhorecky, I., Urschel, J. and Anderson, T. (2000): Resection of invasive pulmonary aspergillosis in immunocompromised patients. Ann. Surg. Oncol., 7, 312-317.

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ORIGINAL ARTICLE

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Polymerase chain reaction assay for specific identification of *Candida guilliermondii* (*Pichia guilliermondii*)

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Abstract The incidence rates of fungemia caused by *Candida guilliermondii* have been increasing over the past several years. Although still relatively rare (1.3% of all cases of fungemia in Japan), most cases of *C. guilliermondii* fungemia occur in patients with cancer or hematological malignancy and their mortality rate is high. As *C. guilliermondii* tends to be resistant to various antifungal agents, early identification of this pathogen and treatment with an appropriate antifungal agent are required to improve survival rates in these patients. However, it is extremely difficult to differentiate *C. guilliermondii* (*Pichia guilliermondii*) from members of the *C. famata* complex. To date, identification based on DNA sequencing has been the only reliable method for the identification of fungal groups. Here, we used a polymerase chain reaction (PCR)-based method that we developed for the simple and reliable identification of *C. guilliermondii* (*P. guilliermondii*). A pair of specific primers was designed corresponding to the 18S rDNA sequence. The PCR system was applied to isolates from fungemia patients. These yeasts could not be identified with CHROMagar Candida, but were successfully identified using this PCR-based system.

Key words *Candida guilliermondii* · Polymerase chain reaction (PCR) · Candidemia · Molecular identification · Candidiasis

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Introduction

The rates of fungal infection due to *Candida* species, known to be agents of opportunistic infectious disease, are showing signs of reaching a plateau.[1] However, the incidence of infection caused by nonalbicans *Candida* has been increasing over the past several years.[2] Nonalbicans *Candida*, as well as *Candida albicans*, occurs in patients with severe underlying conditions. As the patient's clinical condition is often already deteriorating at diagnosis, the usefulness of antifungal agents is limited in such cases. Furthermore, early and accurate identification of pathogenic species has become more important for effective treatment, because susceptibility to antifungal agents varies between species.

The incidence rate of fungemia caused by *Candida guilliermondii* is 1.3% of all cases of candidemia in Japan, and is the sixth highest after *C. albicans* (40.7%), *C. parapsilosis* (23.0%), *C. glabrata* (17.9%), *C. tropicalis* (11.6%), and *C. krusei* (2.4%).[3] However, the incidence of fungemia caused by *C. guilliermondii* has been increasing over the past several years.[4,5] Most cases of invasive candidiasis caused by this pathogen occur in patients with cancer or those with a hematological malignancy,[6,7] as nosocomial infections through the use of a central venous catheter. In addition, this pathogen tends to be innately resistant to various antifungal agents, including azoles, such as fluconazole (FLCZ), and echinocandins, such as micafungin (MCFG).[5,8] Therefore, accurate identification of this pathogen is required for treatment with appropriate antifungal agents. However, this pathogen is morphologically and biochemically indistinguishable from the *C. famata* complex.[7,9–11] Here, we report a polymerase chain reaction (PCR)-based method that we developed for the specific identification of *C. guilliermondii* (*Pichia guilliermondii*), which was used successfully to identify this pathogen from a fungemia patient.

Table 1. Fungal strains used in this study

Species	Strain no.
Candida guilliermondii (*Pichia guilliermondii*)	TIMM0257, 0258, 0260, 1478, 3401, ATCC9058
Candida albicans	TIMM1768, 3400, ATCC10231, 90029
Candida parapsilosis	TIMM0291, 0292, ATCC90018
Candida tropicalis	TIMM0313, 0315, 0325
Candida krusei	TIMM0269, 0271, ATCC6258
Candida glabrata	TIMM1062, 1064, 1065
Candida kefyr	TIMM0300, 0302, 1307
Candida utilis	TIMM0332
Candida dubliniensis	CBS2747, 7988, 8501
Candida auris[12]	JCM15448
Cryptococcus neoformans ver. *neoformans*	TIMM0361, 1316, 1317, 1855
Cryptococcus neoformans ver. *Gattii*	TIMM4905, 1315
Debaryomyces hansenii	TIMM1669, 1670
Pichia anomala	JCM 3585, TIMM3826, 3828
Pichia ohmeri	TIMM3436
Trichosporon beigelii	TIMM3140

Patient, materials, and methods

Organisms and DNA preparation from fungal cells

A total of 43 strains from 16 species of fungi were used in this study (Table 1). All fungal strains were grown on Sabouraud glucose agar at 37°C or CHROMagar Candida (Kanto Chemical, Tokyo, Japan) at 35°C for 24–72 h. Rapid preparation of DNA from the fungi was performed as described by Makimura et al.[13] The concentration of extracted DNA was measured by Quant-it Assay using a Qubit Fluorometer (Invitrogen).

Primer design

Oligonucleotide primers specific for *C. guilliermondii* (*P. guilliermondii*) were designed corresponding to regions of the 18S rRNA gene. The nucleotide sequences of 17 species of fungi, including *C. guilliermondii* (*P. guilliermondii*; Table 2), were obtained from the DDDJ/EMBL/GenBank Database, and the sequences of the 18S rRNA genes were analyzed using GENETYX software (Software Development). Two sites of specific nucleotide sequences of *C. guilliermondii* (*P. guilliermondii*) were determined. The sequences of our designed oligonucleotide PCR primer pair were as follows:

CUGF: 5′-CGGGGAGGTAGTGACAATAC-3′ and CUGR: 5′-CAAACACCACAAGGGCGAAT-3′.

Table 2. DDBJ/EMBL/GenBank accession numbers used for multiple alignment to design *C. guilliermondii* (*P. guilliermondii*)-specific primer pairs

Species	Accession no.
Candida guilliermondii (*P. guilliermondii*)	M60304
Candida albicans	M60302
Candida parapsilosis	M60307
Candida tropicalis	M60308
Candida krusei	M60305
Candida glabrata	M60311
Candida kefyr	M60303
Candida lusitaniae	M60306
Cryptococcus neoformans	M55625
Pichia angusta	M60310
Saccharomyces cerevisiae	V01335
Penicillium chrysogenum	M55628
Blastomyces dermatitidis	M55624
Aspergillus fumigatus	M55626
Pneumocystis carinii	X12708
Coccidioides immitis	M55627
Homo sapiens	M10098

PCR

PCR was performed in reaction mixtures containing 10 µl of 10 × reaction buffer (GE Healthcare) containing 100 µM each of dATP, dCTP, dGTP, and dTTP (Iwaki), 2.5 U of Taq polymerase (GE Healthcare), 30 pmol each primer, and 2 µl of DNA template solution (1.49–13.9 ng/µl). Distilled water was added to a final volume of 100 µl. PCR was performed under the following conditions: 94°C for 5 min; 25 cycles of 94°C for 1 min, 60°C for 15 s, and 72°C for 15 s; and 72°C for 10 min. Distilled water was used as a negative control. The PCR products were separated by electrophoresis (50 V for 45 min) on 1.2% agarose gels, stained with ethidium bromide, and visualized under UV irradiation.

Sensitivity

The sensitivity of the PCR assay with the primer set (CUGF and CUGR) was tested using serial dilutions of DNA solution (100 fg to 100 ng/tube) of *C. guilliermondii* (*P. guilliermondii*) TIMM 0257.

Specificity of primers

The specificity of the PCR primer set (CUGF and CUGR) was tested using all of the fungal strains shown in Table 1.

Clinical specimens

A 57-year-old man was admitted to our hospital for chemotherapy for esophageal cancer in November 2006. The patient had the complication of diabetes. While undergoing chemotherapy, he developed a high fever, which was suspected to be due to infection associated with the central venous catheter. The catheter was removed, and blood and catheter tip cultures were performed. Fungus (yeast) grew from both cultures, and their colonies on CHROMagar Candida (Kanto Chemical) were gray-white in color tinged with red-purple. Identification was impossible because this agar was not applicable for this species. The *C. guilliermondii* (*P. guilliermondii*)-specific PCR method was applied to these two strains. The strains of *C. guilliermondii* (*P. guilliermondii*) TIMM 0257, 3401, 0258, and ATCC 9058 were used as positive controls, and distilled water was used as a negative control. *C. albicans* TIMM 3400 was used as a negative control strain. In addition, we performed biochemical identification using the API 32C system (bioMérieux) for these two species and tested antifungal drug susceptibility by following the standard CLSI (formerly NCCLS) M27-A2 method.[14] To confirm the species identification, we analyzed the sequences of 28S rDNA (D1/D2).[15]

Results

Sensitivity and specificity

A specific product of 200–300 bp was amplified from all 6 strains of *C. guilliermondii* (*P. guilliermondii*) by PCR assay performed using template DNA extracted from the strains shown in Table 1. No products were amplified from the other 37 strains of 15 species examined. Some of the results of the PCR assay for the specificity test are shown in Fig. 1. The PCR assay with the primer set (CUGF and CUGR) was able to detect 10 pg/tube of *C. guilliermondii* (*P. guilliermondii*) genomic DNA with ethidium bromide staining (Fig. 2).

Results of PCR assay of clinical specimens

Figure 3 shows the results of PCR assay using DNA extracted from clinical isolates as the template. The DNA concentrations were: CVno. 3233, 13.9 ng/μl; BLno. 3254, 8.56 ng/μl. Products of 200–300 bp were amplified from both isolates, as well as the positive control, and specific amplification of *C. guilliermondii* (*P. guilliermondii*) was confirmed. In addition, the same results were also obtained when PCR was performed using the colonies directly as the template (colony PCR; Fig. 4). No amplification was seen with *C. albicans* as a negative control strain or with the distilled water negative control.

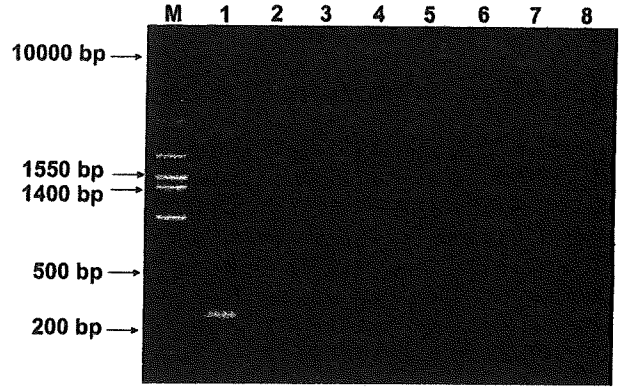

Fig. 2. Sensitivity of the PCR with primer pair CUGF and CUGR. Agarose 1.2% gel electrophoresis of PCR products amplified from various amounts of DNA template from *C. guilliermondii* (TIMM0257). PCR assay was performed using serial dilutions of DNA solution (100 fg to 100 ng/tube) as the template. *Lane M*, marker; *lane 1*, 100 ng/tube; *lane 2*, 10 ng/tube; *lane 3*, 1 ng/tube; *lane 4*, 100 pg/tube; *lane 5*, 10 pg/tube; *lane 6*, 1 pg/tube; *lane 7*, 100 fg/tube; *lane 8*, negative control (distilled water)

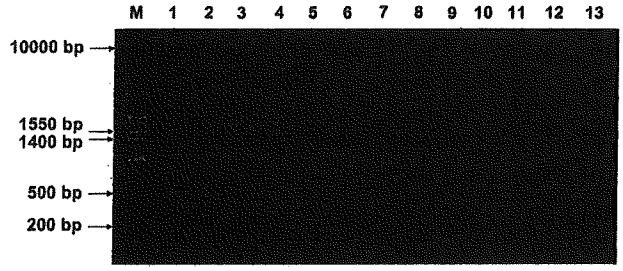

Fig. 1. Specificity of PCR with the primer pair CUGF and CUGR. Agarose 1.2% gel electrophoresis of PCR products amplified from various fungal species. DNA extracted from each strain was used as a template for PCR assay. *Lane M*, marker; *lane 1*, TIMM0257 (*Candida guilliermondii*); *lane 2*, TIMM1768 (*C. albicans*); *lane 3*, CBS2747 (*C. dubliniensis*); *lane 4*, ATCC90018 (*C. parapsilosis*); *lane 5*, TIMM0313 (*C. tropicalis*); *lane 6*, ATCC6258 (*C. krusei*); *lane 7*, TIMM1065 (*C. glabrata*); *lane 8*, TIMM0300 (*C. kefyr*); *lane 9*, JCM15448 (*C. auris*),[12] *lane 10*, TIMM3826 (*P. anomala*); *lane 11*, TIMM0361 (*Cr. neoformans*); *lane 12*, TIMM4905 (*Cr. gattii*); *lane 13*, negative control (distilled water)

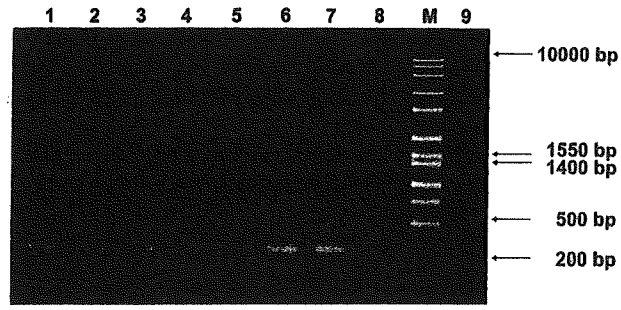

Fig. 3. PCR assay with *C. guilliermondii*-specific primers for fungal strains from clinical isolates. DNA extracted from each strain was used as the template for PCR assay. *Lane 1*, TIMM0257 (*C. guilliermondii*); *lane 2*, TIMM3401 (*C. guilliermondii*); *lane 3*, ATCC9058 (*C. guilliermondii*); *lane 4*, TIMM0258 (*C. guilliermondii*); *lane 5*, TIMM3400 (*C. albicans*); *lane 6*, CVno. 3233 (clinical isolate); *lane 7*, BLno. 3254 (clinical isolate); *lane 8*, negative control (distilled water); *lane 9*, negative control (distilled water); *lane M*, marker

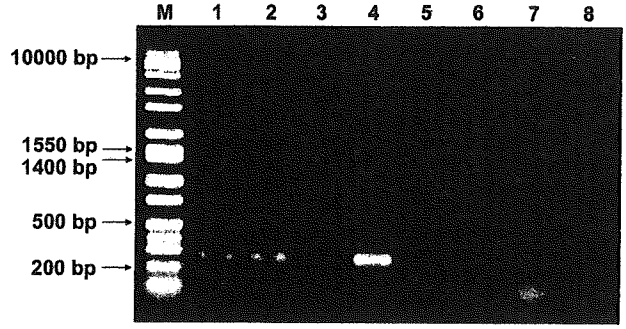

Fig. 4. Colony PCR assay with *C. guilliermondii*-specific primers for fungal strains from clinical isolates. Fungal cells of each colony grown on CHROMagar (Kanto Chemical) were used directly as the template for PCR assay. *Lane M*, marker; *lane 1*, TIMM0257 (*C. guilliermondii*); *lane 2*, TIMM3401 (*C. guilliermondii*); *lane 3*, ATCC9058 (*C. guilliermondii*); *lane 4*, TIMM0258 (*C. guilliermondii*); *lane 5*, TIMM3400 (*C. albicans*); *lane 6*, CVno. 3233 (clinical isolate); *lane 7*, BLno. 3254 (clinical isolate); *lane 8*, negative control (distilled water)

Results of biochemical identification and gene identification

CVno. 3233 and BLno. 3254 were identified as *C. guilliermondii* (*P. guilliermondii*) by the API 32C system. Analysis of the 570-bp 28S rDNA (D1/D2) from both clinical isolates indicated their sequences to be 100% consistent with *P. guilliermondii* (DDBJ/EMBL/GenBank Accession no. U45709).

Susceptibility to antifungal drugs and clinical outcome

CVno. 3233 and BLno. 3254 showed the same susceptibility patterns, with the following MICs for each of the following antifungal drugs: amphotericin-B (AMPH-B), 0.25 μg/ml; flucytosine, <0.125 μg/ml; FLCZ, 0.25 μg/ml; itraconazole, <0.015 μg/ml; miconazole, <0.06 μg/ml; MCFG, 0.5 μg/ml. These two strains were not drug-resistant, and the patient recovered with antifungal therapy using fosfluconazole (200 mg/day, 100 mg/day from day 3, for a total of 2 weeks).

Discussion

The sexual state of *C. guilliermondii* is *P. guilliermondii* or *P. ohmeri*.[16] *C. guilliermondii* is morphologically similar to *C. famata*, the sexual state of which is *Debaryomyces hansenii*, and these species have been treated as *C. famata* complex. In fact, identified *C. guilliermondii* usually includes *P. guilliermondii*, *P. ohmeri*, and *C. famata* (*D. hansenii*), and it is extremely difficult to differentiate between these pathogens.[7,9–11] *P. guilliermondii*, hereinafter described as *C. guilliermondii* (*P. guilliermondii*), included in this complex is the most important pathogenic fungus in humans, and so the early and accurate identification of this pathogen is required for successful treatment.

Agar media for fungi developed to differentiate between pathogenic yeasts in terms of their colony color and morphology on the plates, i.e., Pourmedia Vi Candida (Eiken Kagaku) and CHROMagar Candida (Kanto Chemical), have been widely used in laboratories in Japan. However, *C. guilliermondii* (*P. guilliermondii*) could not be identified on these agar media because its colonies showed a nonspecific color of gray-white tinged with red-purple. In addition, the growth of this pathogen requires incubation for 48 h; although the agar medium easily differentiated the five main yeasts, *C. albicans*, *C. tropicalis*, *C. glabrata*, *C. parapsilosis*, and *C. krusei*, after a 22-h incubation,[17] it is not applicable to this pathogen, which is the sixth most common pathogenic yeast.

Although the API 20C and API 32C systems using biochemical identification have been reported to be able to identify *C. guilliermondii*, they have limitations in their identification capabilities,[7,9] and require significant time and financial costs.[17] The multiplex PCR reported by Carvalho et al.[18] also detected *C. guilliermondii*; however, they did not test the capability of the specific detection of *C. guilliermondii* (*P. guilliermondii*) from the members of the *C. famata* complex.

To reduce laboratory costs, some hospitals only differentiate *C. albicans* from nonalbicans *Candida* and do not perform further differentiation. Several species of nonalbicans *Candida*, such as *C. glabrata*, *C. krusei*, and *C. guilliermondii*, show innate resistance or low sensitivity to antifungal agents.[5,8] *C. guilliermondii* (*P. guilliermondii*) tends to be resistant to various antifungal agents with high MICs against FLCZ and MCFG.[8,19–22] Some strains have cross-resistance to polyenes, e.g., AMPH-B and azoles, e.g., voriconazole.[23–25] Therefore, early and accurate identification of this pathogen is essential for appropriate antifungal treatment. Here, we reported the PCR-based method we developed to be a simple and reliable system for the identification of *C. guilliermondii* (*P. guilliermondii*); to our knowledge, this is the first report regarding the molecular identification of this fungus.

The PCR-based system was applied to an isolate from a patient with fungemia. We suspected this case to involve the sixth most common species of pathogenic yeast, based on the atypical colony features on CHROMagar Candida (Kanto Chemical), and this PCR-based system successfully identified the strain as *C. guilliermondii* (*P. guilliermondii*). In addition, using colony PCR, the yeast can be identified within 140 min. The results from the clinical isolate using this PCR-based system were also supported by those of the API 32C system and DNA sequence analysis.

The lower limit of detection of the PCR assay with CUGF and CUGR was 10 pg/tube, which is sufficient to identify the pathogen obtained from clinical isolates. However, higher sensitivity is required for the PCR assay to be applied directly to clinical specimens.

The pair of specific primers for *C. guilliermondii* (*P. guilliermondii*) was designed as inner-primers of fungi universal primers (B2F and B4R) for PCR diagnosis reported by Makimura et al.[13] The lower detection limit of detection of the PCR assay with B2F and B4R was 1 pg/tube, and it was

able to successfully detect fungal-specific DNA from some clinical specimens (blood, cerebrospinal fluid, and sputum). Therefore, if the nested PCR assay were to be performed with both our primer set (CUGF and CUGR) and the fungi universal primer set (B2F and B4R), users may be able to directly detect *C. guilliermondii* (*P. guilliermondii*) from clinical specimens before isolation of the pathogen.

The PCR system reported here provides a rapid clinically useful method to identify *C. guilliermondii* (*P. guilliermondii*). Further studies of the applicability of the system are underway in our laboratory.

References

1. Kume H, Yamazaki T, Abe M, Tanuma H, Okudaira M, Okayasu I. Epidemiology of visceral mycoses in patients with leukemia and MDS. Analysis of the data in annual registration of pathological autopsy cases in Japan in 1989, 1993, 1997 and 2001. Jpn J Med Mycol 2006;47:15–24.
2. Samonis G, Kofteridis DP, Saloustros E, Giannopoulou KP, Ntziora F, Christidou A, et al. *Candida albicans* versus non-*albicans* bloodstream infection in patients in a tertiary hospital: an analysis of microbiological data. Scand J Infect Dis 2008;40: 414–9.
3. Takakura S, Fujihara N, Saito T, Kudo T, Iinuma Y, Ichiyama S. National surveillance of species distribution in blood isolates of *Candida* species in Japan and their susceptibility to six antifungal agents including voriconazole and micafungin. J Antimicrob Chemother 2004;53:283–9.
4. Pfaller MA, Diekema DJ. Epidemiology of invasive candidiasis: a persistent public health problem. Clin Microbiol Rev 2007;20: 133–63.
5. Pfaller MA, Diekema DJ, Mendez M, Kibbler C, Erzsebet P, Chang SC, et al. *Candida guilliermondii*, an opportunistic fungal pathogen with decreased susceptibility to fluconazole: geographic and temporal trends from the ARTEMIS DISK antifungal surveillance program. J Clin Microbiol 2006;44:3551–6.
6. Girmenia C, Pizzarelli G, Cristini F, Barchiesi F, Spreghini E, Scalise G, et al. *Candida guilliermondii* fungemia in patients with hematologic malignancies. J Clin Microbiol 2006;44:2458–64.
7. Medeiros EA, Lott TJ, Colombo AL, Godoy P, Coutinho AP, Braga MS, et al. Evidence for a pseudo-outbreak of *Candida guilliermondii* fungemia in a university hospital in Brazil. J Clin Microbiol 2007;45:942–7.
8. Pfaller MA, Boyken L, Hollis RJ, Kroeger J, Messer SA, Tendolkar S, et al. In vitro susceptibility of invasive isolates of Candida spp. to anidulafungin, caspofungin, and micafungin: 6 years of global surveillance. J Clin Microbiol 2008;46:150–6.
9. Nishikawa A, Tomomatsu H, Sugita T, Ikeda R, Shinoda T. Taxonomic position of clinical isolates of *Candida famata*. J Med Vet Mycol 1996;34:411–9.
10. Nishikawa A, Sugita T, Shinoda T. Differentiation between *Debaryomyces hansenii/Candida famata* complex and *Candida guilliermondii* by polymerase chain reaction. FEMS Immunol Med Microbiol 1997;19:125–9.
11. Nishikawa A, Sugita T, Shinoda T. Rapid identification of *Debaryomyces hansenii/Candida famata* by polymerase chain reaction. Med Mycol 1999;37:101–4.
12. Satoh K, Makimura K, Hasumi Y, Nishiyama Y, Uchida K, Yamaguchi H. *Candida auris* sp. nov., a novel ascomycetous yeast isolated from the external ear canal of an inpatient in a Japanese hospital. Microbiol Immunol 2009;53:41–4.
13. Makimura K, Murayama SY, Yamaguchi H. Detection of a wide range of medically important fungi by the polymerase chain reaction. J Med Microbiol 1994;40:358–64.
14. National Committee for Clinical Laboratory Standards. Reference method for broth dilution antifungal susceptibility testing of yeasts; approved standard-second edition M27-A2. Wayne, PA: NCCLS; 2002. p. 22 (15).
15. Kurtzman CP, Robnett CJ. Identification of clinically important ascomycetous yeasts based on nucleotide divergence in the 5′ end of the large-subunit(26S). J Clin Microbiol 1997;35:1216–23.
16. Kurtzman CP, Fell JW, Boekhout T, editors. The yeasts: a taxonomic study. 4th ed. Amsterdam: Elsevier Science; 1998. p. 308, 511.
17. Shibata A, Kaneko T, Makimura K, Onozaki M, Ogihara T, Shibata H et al. Comparative study of ability for growth support and species differentiation by colony features on commercial Chromogenic Agar, Pourmedia Vi Candida and CHROMagar Candida. Jpn J Med Mycol 2008;49:33–8.
18. Carvalho A, Costa-De-Oliveira S, Martins ML, Pina-Vaz C, Rodrigues AG, Ludovico P, et al. Multiplex PCR identification of eight clinically relevant Candida species. Med Mycol 2007;45: 619–27.
19. Pfaller MA, Diekema DJ, Rinaldi MG, Barnes R, Hu B, Veselov AV, et al. Results from the ARTEMIS DISK Global Antifungal Surveillance Study: a 6.5-year analysis of susceptibilities of *Candida* and other yeast species to fluconazole and voriconazole by standardized disk diffusion testing. J Clin Microbiol 2005;43:5848–59.
20. Yamaguchi H, Uchida K, Nishiyama Y, Okuzumi K, Oguri T, Adachi K, et al. Nationwide survey of antifungal drug susceptibility of clinical fungal isolates in Japan for the Japan Antifungal Surveillance Program (JASP), 2003. Journal of Japanese Society for Clinical Microbiology 2006;16:13–22.
21. Kobayashi I, Wakasugi M, Ikeda F, Hasegawa M, Suzuki M, Horita H, et al. Susceptibility survey of fresh clinical isolates of *Candida* and *Aspergillus* species to micafungin and seven other antifungal agents. Jpn J Chemother 2006;4:308–14.
22. Dannaoui E, Lortholary O, Raoux D, Bougnoux ME, Galeazzi G, Lawrence C et al. YEASTS Group. Comparative in vitro activities of caspofungin and micafungin, determined using the method of the European Committee on Antimicrobial Susceptibility Testing, against yeast isolates obtained in France in 2005–2006. Antimicrob Agents Chemother 2008;52:778–81.
23. Dick JD, Rosengard BR, Merz WG, Stuart RK, Hutchins GM, Saral R. Fatal disseminated candidiasis due to amphotericin-B-resistant *Candida guilliermondii*. Ann Intern Med 1985;102:67–8.
24. Pfaller MA, Diekema DJ, Messer SA, Boyken L, Hollis RJ, Jones RN; International Fungal Surveillance Participant Group. In vitro activities of voriconazole, posaconazole, and four licensed systemic antifungal agents against *Candida* species infrequently isolated from blood. J Clin Microbiol 2003;41:78–83.
25. Espinel-Ingroff A, Boyle K, Sheehan DJ: In vitro antifungal activities of voriconazole and reference agents as determined by NCCLS methods: review of the literature. Mycopathologia 2001;150: 101–15.

Molecular detection of dermatophytes and nondermatophytes in onychomycosis by nested polymerase chain reaction based on 28S ribosomal RNA gene sequences

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None declared.

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Summary

Background Onychomycosis is often caused by dermatophytes, but the role of nondermatophytes is underestimated due to the difficulty of identifying them by conventional direct microscopy and culture.

Objectives This study aims to detect nondermatophytes, as well as dermatophytes, in the nail samples of patients with onychomycosis using a polymerase chain reaction (PCR)-based culture-independent method.

Materials and methods The nested PCR assay targeting the sequence of the 28S ribosomal RNA gene was used to amplify fungal DNAs from 50 microscopy-positive nail specimens. Newly designed primer sets for dermatophyte universal, Trichophyton rubrum, T. mentagrophytes, Aspergillus spp., Scopulariopsis brevicaulis, Fusarium solani, F. oxysporum, F. verticillioides, Candida albicans and C. tropicalis were used after confirmation of their specificity.

Results Forty-seven cases (94%) were positive for fungal DNA, among which dermatophytes were detected in 39 cases (83·0%): T. rubrum in 35 cases (74·5%) and T. mentagrophytes in eight cases (17·0%). Surprisingly, nondermatophytes were detected in 18 cases (38·3%), both dermatophytes and nondermatophytes in 10 cases (21·3%) and nondermatophytes alone in eight cases (17·0%). Aspergillus spp. alone was observed in five cases (10·6%).

Conclusions This study indicates that most of the affected nail plates of patients with onychomycosis were positive for specific fungal DNAs, and suggests that nondermatophytes detected at high rates may be involved in the pathogenesis of onychomycosis.

Onychomycosis is a general term for any infection of the nail caused by dermatophytes, nondermatophyte moulds or yeasts. On the other hand, tinea unguium refers strictly to a dermatophytic invasion of the nail plate, Trichophyton rubrum being the primary causative organism. The prevalence of onychomycosis in the general population is estimated to range from 0·1% to 11·0% in various countries, and from 6% to 9% in the larger scale surveys.[1,2] Onychomycosis is more common among males and its prevalence increases with age.[1,2] The distinction in terminology between 'onychomycosis' and 'tinea unguium' underscores the fact that, whereas the main causative organisms are the same in each disease, in the former a greater role is assigned to nondermatophyte moulds such as Aspergillus spp., Fusarium spp., Scopulariopsis brevicaulis and Acremonium spp., and yeasts such as Candida albicans, C. tropicalis and C. parapsilosis.

The diagnosis of onychomycosis is conventionally made by direct microscopy[3] and fungal culture; however, culture-based detection methods, including the slide culture, may take a few weeks for definite identification, and may present further difficulties due to occasional, atypical fungal morphology. It is most important to bear in mind regarding the outcome of a fungal culture with onychomycotic specimens that the identification rates are quite low; various studies have found that 15–50% microscopy-positive nail specimens do not result in a positive culture.[4–6] It was therefore uncertain whether the detected organism was the sole pathogen in the affected nail plate. The interaction between dermatophytes and