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SPECIFIC DETECTION OF *ASPERGILLUS* AND *PENICILLIUM* SPECIES FROM RESPIRATORY SPECIMENS BY POLYMERASE CHAIN REACTION (PCR)

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SUMMARY: A polymerase chain reaction (PCR) method capable of detecting both *Aspergillus fumigatus* infections, pulmonary non-*fumigatus* *Aspergillus* species (spp.) and *Penicillium* spp. from clinical specimens was established. The primer pair was designed on the basis of the sequence of the 18S-ribosomal RNA gene of *A. fumigatus* and *P. notatum*. A 385 bp product was successfully amplified by this PCR method from all of 12 medically important *Aspergillus* spp. and *Penicillium* spp. (38 strains), but not from human, calf, *Escherichia coli*, methicillin-resistant *Staphylococcus aureus* (MRSA), any of 14 medically important yeastlike fungal species tested (32 strains) including *Candida albicans*, several non-*albicans* *Candida* and *Saccharomyces cerevisiae*, *Cryptococcus neoformans*, *Mucor* spp. or *Pneumocystis carinii*. This specificity was subsequently confirmed by Southern hybridization analysis. The established PCR can detect such a small amount as 1 pg of *A. fumigatus* DNA by staining the PCR product with ethidium bromide. With sputum specimens from clinically diagnosed aspergilloma patients, this PCR technique was demonstrated to be a more sensitive diagnostic method for *Aspergillus* infections than conventional culture techniques.

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INTRODUCTION

Pulmonary infections caused by various pathogenic or opportunistic fungi, especially *Aspergillus* species (spp.) and *Penicillium* spp., have become increasingly prevalent among immunocompromised patients and even in non-immunocompromised individuals (1-5). Although early diagnosis and therapy can improve the prognosis of such life-threatening fungal infections, the correct antemortem diagnosis by conventional techniques has often been difficult. Treger et al. (6) reported that cultures of respiratory specimens are useful for diagnosis of pulmonary infection caused by aspergilli. Their criterion requires repeated isolation of the same species of the fungi; however, it is well known that the causative fungal agent can be infrequently cultured from sputum specimens and that the cultural examination is laborious and time-consuming. A currently available serodiagnostic kit, Pastrex *Aspergillus* (Diagnostics Pastrex, Paris; 7-9) is limited in clinical usefulness because of low sensitivity and specificity in spite of simplicity and rapidity (4,8,9). In addition, contamination of clinical specimens with various saprophytic filamentous fungi other than aspergilli often give false positive results.

Among recent molecular biological methods developed to improve the sensitivity and specificity for detection of pathogenic aspergillis, Gabal (10) reported that, with the use of total *A. fumigatus* chromosomal DNA probe, *A. fumigatus* DNA extracted from three cfu (neither three cells nor conidia) was detectable. More sensitive detection method will be expected if the polymerase chain reaction (PCR; 11) that is known as the most sensitive and specific technique to detect a specific DNA sequence is used. Spreadbury et al. (12) and Reddy et al. (13) reported PCR conditions specific to *A. fumigatus*. However, the increasing incidence of pulmonary fungal infections caused by non-*fumigatus* aspergilli or *Penicillium* spp. (1-5) has required to develop such a system as can identify a wide range of medically importing fungi. Two different PCR methods capable of detecting a wide range of medically important fungi from clinical specimens have been developed by Hopfer et al. (14) and by us (15). None of these methods, however, can differentiate aspergilli from other fungi contaminating the upper respiratory tract or causing pulmonary infections. The present study was attempted to develop a new PCR primer set specific for aspergilli and penicillia. For this purpose, a primer pair was designed on the basis of DNA sequences existing specifically in the 18S-rRNA gene of *A. fumigatus* and *P. notatum* but not any other major group of pathogenic fungi as the result of search of the GenBank data base. This PCR

system allowed to detect almost exclusively DNAs not only from *A. fumigatus* or *P. notatum* but also from non-*fumigatus* species of *Aspergillus* and *Penicillium* spp. Moreover, sputum specimens collected from three patients with aspergilloma were all test-positive.

MATERIALS AND METHODS

Organisms: The organisms used in this study were shown in Table I.

DNA preparation from fungal cells: All fungal strains are grown on YMPG broth or agar (% w/v; yeast extract, malt extract, and peptone 0.5 each, glucose 1, with or without agar 1.5) at 27 or 37 C for 24 to 48 hr. Small-scale extraction of genomic DNA from yeast-like fungi was performed as described by Philippsen et al (16). For rapid extraction, a small amount of yeast cells grown on YMPG agar was suspended in 100 μ l of lysis buffer (100 mM Tris-HCl pH 7.5, 0.5 % w/v SDS, 30 mM EDTA) and, after mixing with a vortex mixer for 5 sec, the suspension was incubated at 100 C for 15 min and then mixed with 100 μ l of 2.5 M potassium acetate, kept on ice for 60 min, and then centrifuged at 12,000 rpm for 5 min. DNA was precipitated from the supernatant with an equal volume of isopropanol, washed with 0.5 ml of 99% ethanol, dried, and resuspended in 100 μ l of distilled water. A 2- μ l portion of a DNA solution was used as a PCR template.

Small-scale and rapid preparation of DNA from filamentous fungi was performed as described by Bainbridge et al. (17) and Cenis (18), respectively.

E. coli genomic DNA was prepared as described by Sambrook et al (19).

Oligonucleotide design: All oligonucleotides used in this study were designed on the basis of the sequence of the 18S (16S-like) ribosomal RNA gene (18S-rDNA) in the GenBank data base (accession Nos.: M55626, *A. fumigatus*; M55628, *P. notatum*; X54863, *M. racemosus*; M60302, *C. albicans*; M60308, *C. tropicalis*; M60307, *C. parapsilosis*; M60304, *C. guilliermondii*; M60311, *C. glabrata*; M60305, *C. krusei*; M60303, *C. kefyr*; M60306, *C. lusitaniae*; M60310, *H. polymorpha*; V01335, *S. cerevisiae*; M55625, *Cr. neoformans*; X12708, *Pn. carinii*; M24996, *E. coli*; and M10098, *Homo sapiens*), and were searched with the DNASIS (Hitachi Software Engineering Co., Ltd., Yokohama, Japan). Sequences specific for *A. fumigatus* and *P. notatum* but not for other medically important fungi, *E. coli* or *Homo sapiens* were analyzed with the PC/GENE (Intelligenetics Inc., Genofit SA Switzerland, kindly loaned by Teijin Co., Ltd., Yokohama, Japan), and two oligonucleotide primers, B2F: 5'-ACTTTCGATGGTAGGATAG-3' and S3R: 5'-CAGAAGGAAAGGTCCAGCC-3', were made by Genosis Biotechnologies Inc., TX, USA. This primer-pair was expected to amplify a fragment of 385 bp within the region of 18S-rDNA. Additionally, one oligonucleotide probe

Table I. Organisms from which DNA was obtained for use in this study

Organism	Strain
<i>Aspergillus fumigatus</i>	TIMM 0063, 0064, 0068, 0078, 0086, 0090, 0102, 0108, 0109, 1335, 1725, 1732, 1746, 1750, 1770, 1775, 1776, 1778, 1871, 3150, #2021, #2022
<i>A. flavus</i>	TIMM 0057, 0059
<i>A. niger</i>	TIMM 0113, 0114
<i>A. nidulans</i>	TIMM 0111, 2868
<i>A. oryzae</i>	TIMM 0117
<i>A. versicolor</i>	TIMM 0121, 1290
<i>A. terreus</i>	TIMM 0119, 0120
<i>Penicillium expansum</i>	TIMM 1293
<i>P. notatum</i>	TIMM 0883
<i>P. citreo-viride</i>	TIMM 0882
<i>P. commune</i>	TIMM 1331
<i>P. crustosum</i>	TIMM 1332
<i>Mucor circinelloides</i>	TIMM 1324, 1325
<i>M. racemosus</i>	TIMM 1320
<i>Candida albicans</i> serotype A	TIMM 0239, 1623, 1768, 2726
<i>C. albicans</i> serotype B	TIMM 0170, 0172
<i>C. albicans</i> var. <i>stellatoidea</i>	TIMM 0310, 1308
<i>C. tropicalis</i>	TIMM 1312, 0313
<i>C. parapsilosis</i>	TIMM 0288, 0292
<i>C. guilliermondii</i>	TIMM 0257, 0260
<i>C. glabrata</i>	TIMM 1062, 1064
<i>C. krusei</i>	TIMM 0269, 0270
<i>C. kefyr</i>	TIMM 0298, 0302
<i>Hansenula anomala</i>	JCM3585, #0018
<i>H. polymorpha</i>	IFO1166
<i>Saccharomyces cerevisiae</i>	TIMM 0925, 0927
<i>Cryptococcus neoformans</i>	TIMM 0354, 0362, 0372
<i>Pneumocystis carinii</i> ^a	
<i>Escherichia coli</i>	#529
methicillin-resistant <i>Staphylococcus aureus</i> (MRSA) ^b	
calf ^c	
human ^d	

^aDNA was a kind gift from K. Kitada (Department of Tumor Biology, Institute of Medical Science, University of Tokyo), ^bDNA was a kind gift from K. Ubukata (Department of Clinical Pathology, Teikyo University School of Medicine, Tokyo), ^cDNA was purchased from Sigma (St. Louis, MO, U.S.A.),

^dDNA was a kind gift from T. Sakamoto (Department of Obstetrics and Gynecology, Teikyo University School of Medicine).

18SIN3: 5'-CTGAGAAACGGCTACCACAT-3', an internal region within the amplified products, was made for Southern hybridization.

PCR: The reaction mixture for PCR contained 10 μ l of 10 $\times$ reaction buffer [600 mM Tris-HCl pH 8.5, 150 mM (NH₄)₂SO₄, 15 mM MgCl₂], 100 μ M each of dATP, dCTP, dGTP, and dTTP (Pharmacia, Uppsala, Sweden), 2.5 U of Taq polymerase (Pharmacia), 30 pmol of each primer, and a DNA template solution. It was made up to 100 μ l with distilled water and heated to 94 C for 5 min. PCR was performed under the following conditions: 94 C, one minute for denaturation; 57 C, 2 min for primer annealing; and 72 C, 3 min for primer extension; 30 cycles. The last primer extension was carried out for 10 min.

The specificity of this system was tested with samples of genomic DNA extracted from several *Aspergillus* spp. and *Penicillium* spp. to see whether the primer pair amplified the same length of DNA products. Ten nanograms of template DNA was used for each reaction and Southern blot analysis was performed for confirmation.

To determine the lowest limit of detection, PCR was performed the genomic DNA of *A. fumigatus* TIMM3150 serially diluted to give concentrations ranging from one pg to 10 ng.

Clinical specimens: Sputa were taken from three patients with clinically diagnosed as aspergilloma. Circulating galactomannan antigen was detected by Pastrex *Aspergillus* in blood specimens from all three patients, while aspergilli (*A. fumigatus*) were recovered from only one case when culture studies were performed with the sputum specimens. One hundred-microliter aliquots of sputa from these patients were stored at -20 C until needed. DNA extraction was performed as described by Spreadbury et al (12).

Agarose gel electrophoresis and Southern blotting: Five to 10 μ l of PCR product was subjected to electrophoresis in 1.2% agarose gel and visualized by ethidium bromide staining. The resulting DNA bands were transferred from the gel onto nylon membrane (Hybond N+; Amersham, UK) and hybridized with 18SIN3 probe labeled with a chemiluminescence detection system (ECL 3'-oligolabeling and detection system; Amersham). The membranes were washed according to the manufacturer's instructions, and exposed to ISO 20,000 instant film (Polaroid 612 film; Polaroid, Cambridge, MA, USA) for one second to 30 min, with the camera luminometer system (Camlight 501; Analytical Luminescence Laboratory, San Diego, CA).

RESULTS

Design of Oligonucleotide Primer Sequences for PCR and Their Expected Specificity

The primer pair, B2F and S3R, was designed as described in Materials and Methods. Table II shows computer-assisted analysis of B2F and S3R oligonucleotide sequences for their specificity among *A. fumigatus*, *P. notatum*, *C. albicans*, *C. tropicalis*, *Cr. neoformans*, *Pn. carinii*, *M. racemosus* and human. B2F primer sequence was well conserved in all of these medically important fungal species but *M. racemosus*, except for the 3' end of human or *E. coli* 18S-rDNA sequences (data not shown). The S3R primer sequence was so specific for *A. fumigatus* and *P. notatum* that the other fungi or human showed no high homology especially at the 3' region. Since the S3R primer was designed at the spanning helices E21-1 and E21-3 of 18S-rDNA which are lacking in prokaryotes (20), it is natural that this primer would have no sequences homologous with *E. coli* or any other bacteria. It is generally accepted that in PCR the base sequences of the 3' end of the primer play the most critical role in determining the stringency. In other words, if there were mismatched bases at the 3' ends, the PCR primers could not extend efficiently (21) and would fail to amplify the product. It is, therefore, expected that B2F and S3R primer pair could amplify the specific DNA fragment from *A. fumigatus* and *P. notatum* but not that from other fungi, human or bacteria.

Specificity and Sensitivity of PCR in Detecting Fungal DNA

Table III shows the PCR results with the primer pair, B2F and S3R, and template DNAs from a variety of fungi, bacteria and mammals. The specific 385 bp product was amplified only from DNAs from all of the 12 tested *Aspergillus* spp. and *Penicillium* spp. (38 strains). No amplification occurred from DNAs from the other 14 medically important fungal species (32 strains), including *Candida* spp., *S. cerevisiae*, *Hansenula* spp., *Cr. neoformans*, *Pn. carinii*, *Mucor* spp., bacteria, and mammals including human. As shown in Fig. 1A, gel electrophoretic analysis revealed that a single PCR product was amplified with six different *Aspergillus* spp. and *Penicillium* spp., while no PCR product was observed with *M. circinelloides*, *C. albicans*, *Cr. neoformans*, *E. Coli*, or human. No difference in size of the amplified product was found among various species of aspergilli or penicillia, nor was amplification observed without templates (Lane 1, Fig. 2). Subsequent Southern blotting confirmed the high specificity of this PCR system for aspergilli and penicillia (Fig. 1B).

Table II. Nucleotide sequences of B2F and S3R primer-region in the 18S-rRNA gene of several fungi and human

Organism	B2F		S3R	
	5'	ACTTT-CGATGGTAGGATAG 3'	5'	CAG-AAGGAAAGGTCAGC--C 3'
<i>Aspergillus fumigatus</i>	5'	 3'	5'	 3'
<i>Penicillium notatum</i>	5'	 3'	5'	 3'
<i>Candida albicans</i>	5'	 3'	5'	CT.G..TG- 3'
<i>Candida tropicalis</i>	5'	 3'	5'	CT.G.TTG- 3'
<i>Cryptococcus neoformans</i>	5'	 3'	5'	G..T.....A 3'
<i>Pneumocystis carinii</i>	5'	 3'	5'	G..G.....AT...T..T 3'
<i>Mucor racemosus</i>	5'	TGG...GA...T...A..... 3'	5'	...A.A--...A.C.TC.AT.- 3'
<i>Homo sapiens</i>	5'	TCGCC 3'	5'	.C.AG...C.....GG.G.GGA. 3'

•: conserved base, -: gap base.

Table III. Results of PCR analysis of a variety of organisms

Positive	Negative
<i>Aspergillus fumigatus</i>	<i>Mucor circinelloides</i>
<i>Aspergillus flavus</i>	<i>Mucor racemosus</i>
<i>Aspergillus niger</i>	<i>Candida albicans</i> serotype A and B
<i>Aspergillus terreus</i>	<i>Candida albicans</i> var. <i>stellatoidea</i>
<i>Aspergillus versicolor</i>	<i>Candida tropicalis</i>
<i>Aspergillus oryzae</i>	<i>Candida parapsilosis</i>
<i>Aspergillus nidulans</i>	<i>Candida glabrata</i>
<i>Penicillium commune</i>	<i>Candida guilliermondii</i>
<i>Penicillium expansum</i>	<i>Candida krusei</i>
<i>Penicillium notatum</i>	<i>Candida kefyr</i>
<i>Penicillium curstosum</i>	<i>Saccharomyces cerevisiae</i>
<i>Penicillium citreo-viride</i>	<i>Hansenula anomala</i>
	<i>Hansenula polymorpha</i>
	<i>Cryptococcus neoformans</i>
	<i>Pneumocystis carinii</i>
	<i>Staphylococcus aureus</i> (MRSA)
	<i>Escherichia coli</i>
	calf
	human

Fig. 1. Specificity of the PCR with primer pair, B2F and S3R. Agarose (1.2%) gel electrophoresis was done with PCR products amplified with 10 ng of genomic DNA from various organisms as the template. (A): Stained with ethidium bromide and visualized by UV irradiation, (B): Southern blotting done by the chemiluminescence method. Photographs were taken with Polaroid 612, ISO 20,000 film. Lanes: M; *Hind*III-digested lambda DNA, 1: *A. fumigatus* TIMM3150, 2: *A. flavus* TIMM0057, 3: *A. niger* TIMM0113, 4: *A. terreus* TIMM0119, 5: *P. commune* TIMM1331, 6: *P. expansum* TIMM1293, 7: *M. circinelloides* TIMM1324, 8: *C. albicans* TIMM1768, 9: *Cr. neoformans*, 10: *E. coli*, 11: human.

Fig. 2. Sensitivity of the PCR with primer pair, B2F and S3R. Agarose (1.2%) gel electrophoresis was done with PCR products amplified with various amounts of *A. fumigatus* genomic DNA. Stained with ethidium bromide and visualized by UV irradiation. Lanes: M: *Hind*III-digested lambda DNA, 1-6: 0, 1 pg, 10 pg, 100 pg, 1 ng, and 10 ng of *A. fumigatus* TIMM3150 DNA, respectively.

Figure 2 shows that the PCR system with the primer pair B2F and S3R is so sensitive that an amount as small as 1 pg or less of genomic *A. fumigatus* DNA was detectable by ethidium bromide staining and UV visualization. This DNA level is equivalent to that of approximately 10 cells of *A. fumigatus*, as well as of *A. nidulans* (22) and *A. niger* (23), on an assumption that genome size of *A. fumigatus* is similar to that of the latter two species.

Detection of Specific DNA by PCR from Clinical Specimens

The specific 385 bp DNA fragment was successfully amplified when PCR was run with the use of DNA extracted from sputum specimens as the template in all of three cases of clinically diagnosed aspergilloma. The specimen from case 1 which yielded *A. fumigatus* growth was demonstrated to be PCR-positive; the specific 385 bp product was distinguishable under UV visualization (Lane 1, Fig. 3A) and confirmed to be the target DNA by subsequent Southern blotting (Lane 1, Fig. 3B). Although the specimens from cases 2 and 3 were culture negative, they

Fig. 3. Detection of amplified products by PCR with primer pair, B2F and S3R with the use of DNA extracted from sputa collected from three patients clinically suspected of having pulmonary aspergilloma. Agarose (1.2%) gel electrophoresis of PCR products amplified. Lanes: M: *Hind*III-digested lambda DNA; 1: case 1 with positive culture (*A. fumigatus*); 2: case 2 with negative culture; 3: case with negative culture; 4: 10 ng of *A. fumigatus* DNA.

gave stronger bands of the PCR product (Lanes 2 and 3, Fig. 3A), whose specificity was confirmed by subsequent Southern blotting (Lanes 2 and 3, Fig. 3B). Some DNA fragments different from the specific 385 bp fragment were also amplified with DNA from the case 3 specimen (Lane 3, Fig. 3). However, none of these extrafragments hybridized with the internal DNA probe, 18SIN3, indicat-

ing that they were products resulting from nonspecific amplification (Lane 3, Fig. 3).

DISCUSSION

Using the 18S-rDNA based primer pair, we established a new PCR system for specific detection of genomic DNA from *Aspergillus* spp. and *Penicillium* spp. Its specificity and sensitivity are high enough, so the diagnosis of pulmonary aspergillosis with DNA as the template extracted from sputum specimens is practically useful.

There are publications reporting the species-specific PCR-diagnostic methods for fungal infections; however, their applicability is limited to *A. fumigatus* (12,13), *C. albicans* (24-28) and *Pn. carinii* (29-33), the latter being recently viewed as belonging to fungi rather than protozoa (34). Although *A. fumigatus* is the major causative agent of pulmonary aspergillosis, there is an increase in incidence of respiratory infections caused by several non-*fumigatus* *Aspergillus* spp., such as *A. flavus*, *A. niger*, and *A. terreus*, and, at a lesser frequency, the *Penicillium* spp., such as *P. marneffei* and *P. commune* (1-5), among immunocompromised patients. This spurred us to develop a new PCR diagnostic method applicable to all species of *Aspergillus*.

DNA sequences of the 18S-rDNA of various organisms including fungi have been deposited in the GenBank data base primarily for the purposes of phylogenetic study (20,34-36). Using the primer pair which was designed on the basis of conserved sequences of 18S-rDNA, Hopfer et al. (15) and the authors (16) developed PCR techniques to detect a wide range of fungal species from clinical specimens. Although these PCR methods are useful for screening patients with fungal infections, more specific PCR diagnostic method(s) like the one reported in this paper is needed to discriminate aspergillosis from other infections to ensure the correct diagnosis.

Sputum, although frequently and heavily contaminated with various microorganisms, is a suitable specimen for both culture studies. The PCR technique to detect the causative agent of respiratory infections because sputum specimen can be obtained easily and non-invasively. Actually, sputa have been favorably used for PCR methods to detect various microorganisms including *Pn. carinii* (29-33).

We therefore chose sputa as a clinical specimen with which pulmonary aspergillosis can be satisfactorily diagnosed by our PCR method.

In the present study, we extracted DNA from sputa by the method described by Spreadbury et al (12). DNA thus prepared appeared to be more sensitive in our PCR test than that prepared by the Buchman's method (24) which we used in our previous study (15; data not shown). Our assumption is that the digestive procedures with DNase used in Buchman's method to eliminate human DNA also digest fungal DNA in the specimen. With DNA extracted by Spreadbury's method (12), the specific DNA fragments were amplified by our PCR system with higher sensitivity than the conventional culture method from all three sputa taken from clinically diagnosed and circulating galactomannan antigen-positive aspergillosis patients.

Cases of secondary *Aspergillus* spp. infections which frequently occur in immunocompromised patients taking preventive or maintenance therapy with fluconazole or other oral azoles are now increasing (4). Although systemic penicilliosis usually occurs much less frequently, *P. marneffei* infections have now become a serious problem among AIDS patients in Indonesia, Thailand and some other South Asian countries (1,5). Once immunocompromised patients are infected with pathogenic *Aspergillus* or *Penicillium*, serious consequences often develop with a fatal outcome unless adequate antifungal chemotherapy is started at the initial stage of infection. The PCR diagnostic system would favorably support the early diagnosis of invasive aspergillosis and penicilliosis. Further evaluation of this PCR method is underway with other clinical specimens and a large number of clinical cases.

For epidemiological study, identification at the species level may be required. Since 18S-rDNA sequences of many fungi are known, analysis of species-specific sequences contained in our PCR products enables us to differentiate or identify species. Restriction enzyme analysis, Southern blotting, nested PCR analysis and other means are helpful in this context, and these studies are also underway in our laboratory.

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