Detection of a wide range of medically important fungi by the polymerase chain reaction

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Summary. A polymerase chain reaction (PCR) method was developed that was capable of detecting a wide range of medically important fungi from clinical specimens. The primer pair was designed in conserved sequences of 18S-ribosomal RNA genes shared by most fungi. The lower limit of detection of this PCR technique was 1 pg of Candida albicans genomic DNA by ethidium bromide staining and 100 fg after Southern analysis. A 687-bp product was amplified successfully by PCR from all 78 strains of 25 medically important fungal species studied, including Candida spp., Hansenula spp., Saccharomyces cerevisiae, Cryptococcus neoformans, Trichosporon beigelii, Malassezia furfur, Pneumocystis carinii, Aspergillus spp., and Penicillium spp., but not from any strains of Mucor spp., Escherichia coli, or methicillin-resistant Staphylococcus aureus (MRSA), calf thymus or human placenta. This specificity was subsequently confirmed by Southern analysis. PCR analysis of blood specimens collected from mice systemically infected with C. albicans and clinical samples including blood, cerebrospinal fluid and sputum appeared to be a more sensitive diagnostic method for invasive fungal infections than a conventional blood culture technique.

Introduction

Invasive candidosis, aspergillosis and other life-threatening fungal infections occur increasingly frequently in immunocompromised patients. Furthermore, the variety of causative agents is expanding. These factors make laboratory diagnosis by conventional culture methods more difficult and time-consuming. Serodiagnostic kits have been developed for early diagnosis, but some of these have problems in sensitivity or specificity, or both, even though they are rapid to perform.

To improve the sensitivity and specificity of detection of pathogenic fungi, molecular biological methods have been developed recently. Some laboratory diagnostic methods with Southern hybridisation have been reported. Gabal detected A. fumigatus DNA in an amount equivalent to 3 cfu with a total Aspergillus fumigatus chromosomal DNA probe. Holmes et al. reported that Candida albicans specific repetitive sequence was detectable from human blood specimens that contained ≥ 500 yeast cells/ml. DNA probes for ribosomal RNA (rRNA) of several species of pathogenic fungi are now available commercially.

The polymerase chain reaction (PCR) is the most sensitive and specific technique of detecting a specific DNA sequence, and species-specific PCR-diagnostic methods applicable to C. albicans, A. fumigatus or Pneumocystis carinii are available. These PCR techniques are capable of detecting > 1 pg of fungal genomic DNA or one-to-15 fungal cells with great accuracy.

The increasing incidence of invasive fungal infections caused by uncommon fungi including non-albicans Candida spp., Hansenula spp., Saccharomyces cerevisiae, Trichosporon beigelii, Malassezia furfur and Penicillium spp., in immunocompromised hosts has made it essential to have a means of identifying a broad variety of medically important fungi. Hopfer et al. reported a PCR method with a previously described primer system to meet this need and attempted to differentiate species with restriction analysis of amplified products. However, as the primer system they used was developed originally for the study of fungal phylogenetics, it is questionable whether the system is also applicable to medically important fungi. In the present study a new PCR primer system was used that had broad detection capability with a primer pair based on conserved DNA sequences of 18S-rRNA genes of medically important fungi in the GenBank data base. This system was applied to blood specimens from a murine model of invasive candidosis and to clinical specimens from human patients.
Organisms

The following strains were used in this study: C. albicans serotype A TIMM nos. 0239, 1623, 1768, 2726; C. albicans serotype B TIMM nos. 0170, 0172; C. albicans var. stellatoidea TIMM nos. 0310, 1308; C. tropicalis TIMM nos. 1312, 0313; C. parapsilosis TIMM nos. 0288, 0292; C. guilliermondii TIMM nos. 0257, 0260; C. glabrata TIMM nos. 1062, 1064; C. krusei TIMM nos. 0269, 0270; C. kefyr TIMM nos. 0298, 0302; H. anomala JCM3585, #0018; H. polymorphaIFO1166; S. cerevisiae TIMM nos. 0925, 0927; C. neoformans TIMM nos. 0354, 0362, 0372; T. beigeli TIMM nos. 1287, 1526, 1573; M. farfur TIMM nos. 1847, 1848, 1850, 1851, 1852, 2462, 2535, 2681, 2718, 2782; A. fumigatus TIMM nos. 0063, 0064, 0068, 0078, 0086, 0090, 0210, 0108, 0109, 1335, 1725, 1732, 1746, 1750, 1770, 1775, 1776, 1871, 3150, #2021, #2022; A. flavus TIMM nos. 0057, 0059; A. niger TIMM nos. 0113, 0114; A. nidulans TIMM nos. 0111, 2868; A. oryzae TIMM nos. 0117; A. versicolor TIMM nos. 0121, 1290; A. terreus TIMM nos. 0119, 0120; P. expansum TIMM nos. 1293; P. notatum TIMM nos. 0883; P. citreo-viride TIMM nos. 0882; P. commune TIMM nos. 1331; P. crustosum TIMM nos. 1332; Mucor circinelloides TIMM nos. 1324, 1325; Mucor racemosus TIMM nos. 1320 and Escherichia coli #529. Other genomic DNA samples provided by the following laboratories were also used: Pn. carinii from K. Kitada, Department of Tumor Biology, Institute of Medical Science, University of Tokyo; methicillin-resistant Staphylococcus aureus (MRSA) from K. Ubukata, Department of Clinical Pathology, Teikyo University School of Medicine, Tokyo; calf thymus (Sigma); and human placenta from T. Sakamoto, Department of Obstetrics and Gynecology, Teikyo University School of Medicine.

DNA preparation from fungal cells

All fungal strains were grown in or on YMPG broth or agar (yeast extract 0.5% w/v, malt extract 0.3% w/v, peptone 0.5% w/v, glucose 1% w/v, with or without agar 1.5% w/v) at 27°C or 37°C for 1–2 days.

Small scale extraction of genomic DNA from yeast-like fungi was performed as described by Philipssen et al.26 For rapid extraction of DNA from yeast-like fungi, a small amount of the yeast colony was suspended in 100 μl of lysis buffer (100 mm Tris-HCl, pH 7.5, SDS 0.5% w/v, 30 mm EDTA). After vortex mixing for 5 s, this mixture was incubated at 100°C for 15 min; 100 μl of 2.5 M potassium acetate was added and it was mixed again, incubated on ice for 60 min, centrifuged at 12000 rpm for 5 min and the supernate was transferred to a new tube. DNA was precipitated with an equal volume of isopropanol, washed with 0.5 ml of ethanol 99%, dried and resuspended in 100 μl of distilled water. The PCR template was 2 μl of this DNA solution.

Small scale extraction of DNA from filamentous fungi was performed as described by Bainbridge et al.28 and rapid extraction as described by Cenis.87 E. coli genomic DNA was extracted as described by Sambrook et al.28

Oligonucleotide design

The design of oligonucleotides used in this study was based on comparison of the sequences of 18S (16S-like) ribosomal RNA genes (rDNA) in the GenBank data base (accession nos: 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; M55626, A. fumigatus; M55628, P. notatum; M55624, Blastomyces dermatitidis; M55627, Coccidioides immitis; X54863, Muc. racemosus; M24996, E. coli; M10098, Homo sapiens). The highly conserved sequences of the medically important fungi, but not of Muc. racemosus, E. coli or H. sapiens were analysed with the PC/GENE (IntelliGenetics Inc., Genofit. SA, USA, kindly loaned by Teijin Co., Ltd, Yokohama, Japan) and two oligonucleotide primers-B2F: 5'-ACT-TTCGATGTTAGGATAG-3' and B4R: 5'-TGATC-GTCTTGGATCCCTCA-3'—made by Genosis Biotechnologies Inc., Texas, USA. The primers were expected to amplify a fragment of 687 bp within the 18S-rDNA. Also, one oligonucleotide probe 18SIN3:
Fig. 1. Specificity of the PCR with primer pair B2F and B4R. Agarose 1.2% gel electrophoresis of PCR products amplified from 10 ng of genomic DNA templates from various organisms was done. A, stained by ethidium bromide and visualised by UV irradiation; B, followed by the chemiluminescence method of Southern analysis detected by Polaroid 612, ISO 20000 film. Lanes: M, HindIII-digested lambda DNA; 1, A. fumigatus TIMM3150; 2, A. flavus TIMM0057; 3, A. niger TIMM0113; 4, P. commune TIMM1331; 5, C. albicans TIMM1768; 6, C. parapsilosis TIMM0292; 7, C. tropicalis TIMM0313; 8, Cr. neoformans TIMM0354; 9, Muc. circinelloides TIMM1324; 10, E. coli; 11, human.

5'-CTGAGAAACGGCTACCACAT-3'. an internal region within the amplified products, was made for Southern hybridisation.

**PCR**

Each PCR assay contained 10 µl of 10 x reaction buffer [600 mM Tris-HCl, pH 8.5, 150 mM (NH₄)₂SO₄, 15 mM MgCl₂], 100 µM each of dATP, dCTP, dGTP and dTTP (Pharmacia, Uppsala, Sweden), 2.5 U of Taq polymerase (Pharmacia), 30 pmol of each primer and DNA template solution. The volume was made up to 100 µl with distilled water. Each mixture was heated to 94°C for 5 min and PCR was performed under the following conditions: 94°C for 1 min; 55°C for 2 min and 72°C for 3 min; 30 cycles. Thermal cycles were terminated by polymerisation at 72°C for 10 min.

To examine the specificity of this system, the samples of genomic DNA extracted from the organisms were tested to see whether the primer pair amplified the
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Fig. 2. Sensitivity of the PCR with primer pair B2F and B4R. Agarose 1.2% gel electrophoresis of PCR products amplified from different amounts of C. albicans, E. coli and human genomic DNA template was done. A, stained by ethidium bromide and visualised by UV irradiation; B, detection by chemiluminescence; detected by Polaroid 612, ISO 20000 film. Lanes: M, HindIII-digested lambda DNA; 1-7, 0, 100 fg, 1 pg, 10 pg, 100 pg, 1 ng, 10 ng of C. albicans TIMM2726 DNA; 8, 10 ng of E. coli genomic DNA; 9, 10 ng of human genomic DNA.

Infection of mice with C. albicans

On day 0, eight of 10 female ICR mice (5 weeks old; obtained from Nippon Bio-Supp. Center, Tokyo, Japan) were given 10⁶ cells of C. albicans TIMM1768 by intravenous injection in 200 μl of sterile saline. Two mice were killed 1–4 days after inoculation, dissected and blood samples were taken from the hearts, to which 5 μl of 0.5 M EDTA was added. From each blood sample, 50 μl was cultured on YMPG agar incubated at 37°C for 2 days. Remaining blood samples were stored immediately at −80°C until needed. DNA was extracted from 100 μl of the blood.
sample from each mouse as described by Buchman et al.\textsuperscript{11}

Clinical specimens

Blood, cerebrospinal fluid or sputum was taken from immunocompromised patients with fever who did not respond to any antibacterial chemotherapy and 100-\(\mu\)l volumes of the samples were stored at \(-80^\circ\)C until needed. DNA extraction was performed as described by Buchman et al.\textsuperscript{11}

Serodiagnostic tests

Cand-Tec\textsuperscript{4} (Ramco, Houston); Pastorex Aspergillus\textsuperscript{5} (Diagnostics Pastorex, Paris); Serodirect Eiken Cryptococcus\textsuperscript{5} (Eiken Kagaku Corp., 1-33-8 Hongo, Bunkyo-ku, Tokyo 113 Japan) and G test\textsuperscript{6} (Seikagaku Corp., 2-1-5 Hashimoto-cho, Nihombashi, Chu-ku, Tokyo 103 Japan) were used as shown in table 111.

Agarose gel electrophoresis and Southern analysis

PCR product (5–10 \(\mu\)l) was electrophoresed in agarose 1.2\% gel and visualised by staining with ethidium bromide. The DNA was transferred from the gels on to nylon membranes (Hybond N\textsuperscript{+}; Amerham), and then hybridised with the 18SIN3 probe labelled with a chemiluminescence detection system (ECL 3’-oligolabelling and detection system; Amerham). The membranes were washed according to the manufacturer’s instructions and exposed to ISO 20000 instant film (Polaroid 612 film; Polaroid, Cambridge, USA) for 1 s–30 min in the camera luminometer system (Camlight 501; Analytical Luminescence Laboratory, San Diego, CA, USA).

Results

Specificity of detection of fungi by PCR

A product of c. 700 bp was amplified by PCR from all 78 strains of 25 medically important fungal species tested (table I), but not from \textit{Muc. circinelloides}, \textit{Muc. racemosus}, \textit{E. coli}, MRSA, calf thymus or human placenta. No amplification was observed without templates. Fig. 1A shows PCR products amplified from eight different fungi, but not from \textit{Muc. circinel-}

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<th>Days after infection</th>
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\textsuperscript{*}Not infected.

E. coli or man. No differences in length were found among any of the species. This specificity was subsequently confirmed by Southern analysis (fig. 1B).

Sensitivity of detection of \textit{C. albicans} by PCR

PCR with the primer pair B2F and B4R was able to detect 1 pg of \textit{C. albicans} genomic DNA with ethidium bromide staining (fig. 2A) and 100 fg by chemiluminescence (fig. 2B). This sensitivity is equivalent to the detection of one-to-two yeast cells.\textsuperscript{24} PCR with the template extracted from blood samples containing \textit{C. albicans} cells detected 10\textsuperscript{2} \textit{C. albicans} cells by ethidium bromide staining and 10 cells by Southern analysis (data not shown). There was no amplified product from blood without \textit{C. albicans} cells and there was no cross-hybridisation to the probe 18SIN3.

Detection of fungal-specific DNA in blood specimens from mice infected with \textit{C. albicans}

A summary of the PCR and culture results and the presence of kidney abscesses is given in table II. Only one sample on day two after infection (mouse no. 3) showed a positive culture. The culture-positive sample was also positive by PCR, and five of seven culture-negative samples also resulted in a positive PCR result. In every mouse from day two to day four, multiple foci of kidney abscesses were observed. Since the intensities of PCR products from culture-negative samples were as strong as those from culture-positive samples and the results of PCR, culture and detection of kidney abscesses were all negative in uninfected mice, this does not appear to be an indication of false positive results. All PCR products led to amplification of single fragments c. 700 bp in length, and they were confirmed as target DNA by Southern analysis.

Detection of fungal-specific DNA from clinical specimens

A summary of the results of PCR, culture and diagnostic kits is given in table III. The samples from patients 1, 2 and 3 were culture-positive: \textit{C. albicans} from blood; \textit{H. anomala} from cerebrospinal fluid and \textit{A. fumigatus} from sputum, respectively; all of them gave positive results in the PCR. Samples from patients no. 4 were PCR positive; the blood culture was negative, but CSF was culture positive for \textit{Cr. neoformans}. Blood samples from patients 5, 6 and 7 (immunocompromised hosts who were resistant to antibacterial antibiotics) gave negative results in culture, but two of the three were positive in the PCR. Each amplified DNA fragment revealed a single band of c. 700 bp and these bands were confirmed as target DNA by Southern analysis.

Discussion

The results of this study demonstrated firstly that the 18S-rDNA-based PCR method had high sensitivity and specificity for a wide range of medically important
fungi. Secondly, the capability of this technique to identify these fungi from the blood of an animal model and clinical specimens of blood, cerebrospinal fluid and sputum make it useful for clinical diagnosis.

The sensitivity of this PCR system with the primer pair B2F and B4R, with both purified DNA and yeast cells contained in blood reached a detection level as high as that of the previously reported PCR method for pathogenic fungi.11-15 When purified DNA or cultured cells of *C. albicans* were used as the templates. There are only three reports11,15 referred to the sensitivity of PCR with artificial samples containing body fluids and the lower limit was the same as that reported here: 10-100 cells or cfu prepared from blood or urine. Moreover, from the results of the animal model and clinical specimens, PCR was shown to be more sensitive than culture methods with 0.1 ml of blood samples. This may be explained by the loss of viability of fungi contained in blood, but > 10^6 cells are estimated to exist in 0.1 ml of blood if calculated as DNA. In this sensitivity study, two kinds of PCR templates were used, the purified DNA solution and crude DNA extracts from blood containing *C. albicans* cells. Assuming that one *C. albicans* cell contains c. 37 fg of DNA,29 Buchman's method of fungal DNA preparation from clinical samples including blood may be highly efficient.

DNA sequences of 18S-rDNA of various organisms have been deposited in the GenBank data base for purposes of phylogenetic study.34,35,31 Our primer pair, B2F and B4R, was designed in conserved sequences of 18S-rDNA to be specific only for medically important fungi and this specificity was achieved. For example, there have been numerous reported cases of systemic infection with *M. furfur*32 and this was detected successfully by the PCR system although the organism cannot be detected by a conventional culture system. *Pn. carinii* (which was recently demonstrated to be a fungus)39 was also detected by this PCR system. Some reports of detection of *Pn. carinii* with PCR have been published.16-20 Lipschik et al.14 reported the PCR diagnosis of *Pn. carinii* infection based on 18S-rDNA different from that used here. Their primer pair was designed to have species-specificity as reported by many others.13,14,17,20 Diagnostic kits also reveal less sensitivity on tested samples than by our technique.

New technology, such as enzymic detection of PCR products22 and the non-radioisotopic method of Southern analysis as demonstrated in this study, make PCR applicable to the routine laboratory diagnosis of infections by medically important fungi. Further evaluation of this PCR method with other clinical specimens is underway.

We thank K. Kitada, University of Tokyo, K. Ubukata and T. Sakamoto, Teikyo University School of Medicine, for providing DNA, M. Mituya and K. Wada, Department of Microbiology and Immunology, Teikyo University School of Medicine, for serodiagnostic kit analysis; and K. Uchida, Research Center for Medical Mycology, Teikyo University, for providing fungal strains. We also thank the various hospital colleagues who supplied us with clinical specimens.

### References


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