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

K. MAKIMURA*, SOMAY Y. MURAYAMA* and H. YAMAGUCHI*†

*Department of Microbiology and Immunology, Teikyo University School of Medicine, 2-11-1 Kaga, Itabashi-ku, Tokyo 173 and †Research Center for Medical Mycology, Teikyo University, 359 Ohtsuka, Hachioji, Tokyo 192-03, Japan

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.[1–3] 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.[4–6]

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[7] detected *A. fumigatus* DNA in an amount equivalent to 3 cfu with a total *Aspergillus fumigatus* chromosomal DNA probe[1]. Holmes *et al.*[8] 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.[9]

The polymerase chain reaction (PCR[10]) 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.[11–20] 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[1–3,21,22] has made it essential to have a means of identifying a broad variety of medically important fungi. Hopfer *et al.*[23] reported a PCR method with a previously described primer system[24] 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.

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Table I. Fungal strains detected by PCR with the primer pair B2F and B4R

Strain	Number of strains tested
C. albicans serotype A	4
C. albicans serotype B	2
C. albicans var. *stellatoidea*	2
C. tropicalis	2
C. parapsilosis	2
C. guilliermondii	2
C. glabrata	2
C. krusei	2
C. kefyr	2
H. anomala	2
H. polymorpha	1
S. cerevisiae	2
Cr. neoformans	3
T. beigelii	3
M. furfur	10
Pn. carinii	1
A. fumigatus	22
A. flavus	2
A. niger	2
A. nidulans	2
A. oryzae	1
A. terreus	2
P. expansum	1
P. citreo-viride	1
P. commune	1
P. notatum	1
P. crustosum	1

Materials and methods

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. polymorpha* IFO1166; *S. cerevisiae* TIMM nos. 0925, 0927; *Cr. neoformans* TIMM nos. 0354, 0362, 0372; *T. beigelii* TIMM nos. 1287, 1526, 1573; *M. furfur* 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, 1778, 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; *Muc. 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 0·3% w/v, malt extract 0·3% w/v, peptone 0·5% w/v, glucose 1%, with or without agar 1·5% w/v) at 27° or 37°C for 1–2 days.

Small scale extraction of genomic DNA from yeast-like fungi was performed as described by Philippsen *et al.*[25]

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.*[26] and rapid extraction as described by Cenis.[27]

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′-ACTTTCGATGGTAGGATAG-3′ and B4R: 5′-TGATCGTCTTCGATCCCCTA-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:

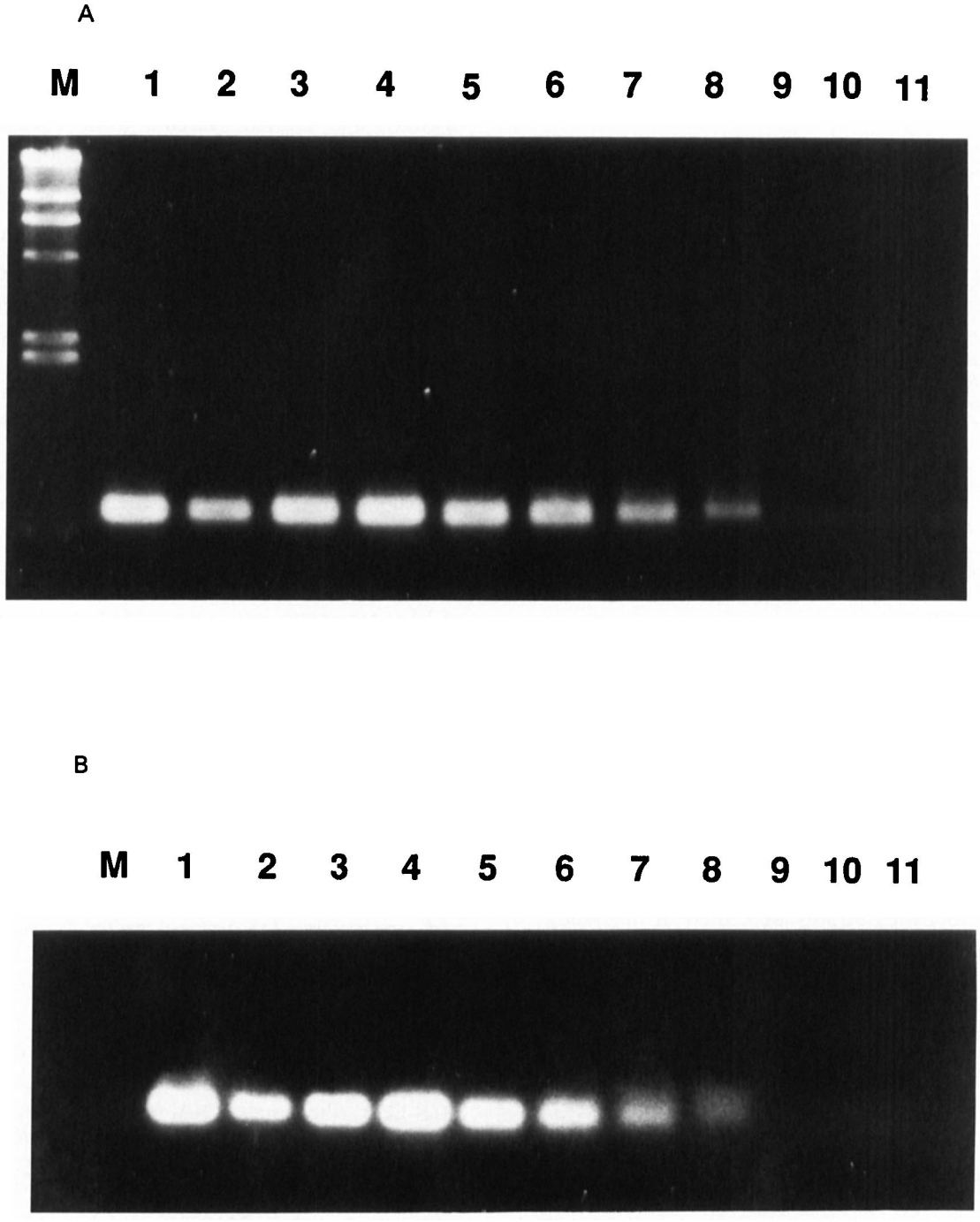

Fig. 1. Specificity of the PCR with primer pair B2F and B4R. Agarose 1·2% gel electrophoresis of PCR products amplified from 10 ng of genomic DNA templates from various organisms was done. **A**, stained by ethidium bromide and visualised by UV irradiation; **B**, followed by the chemiluminescence method of Southern analysis detected by Polaroid 612, ISO 20000 film. Lanes: **M**, *Hin*dIII-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 × reaction buffer [600 mM Tris-HCl, pH 8·5, 150 mM $(NH_4)_2SO_4$, 15 mM $MgCl_2$], 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

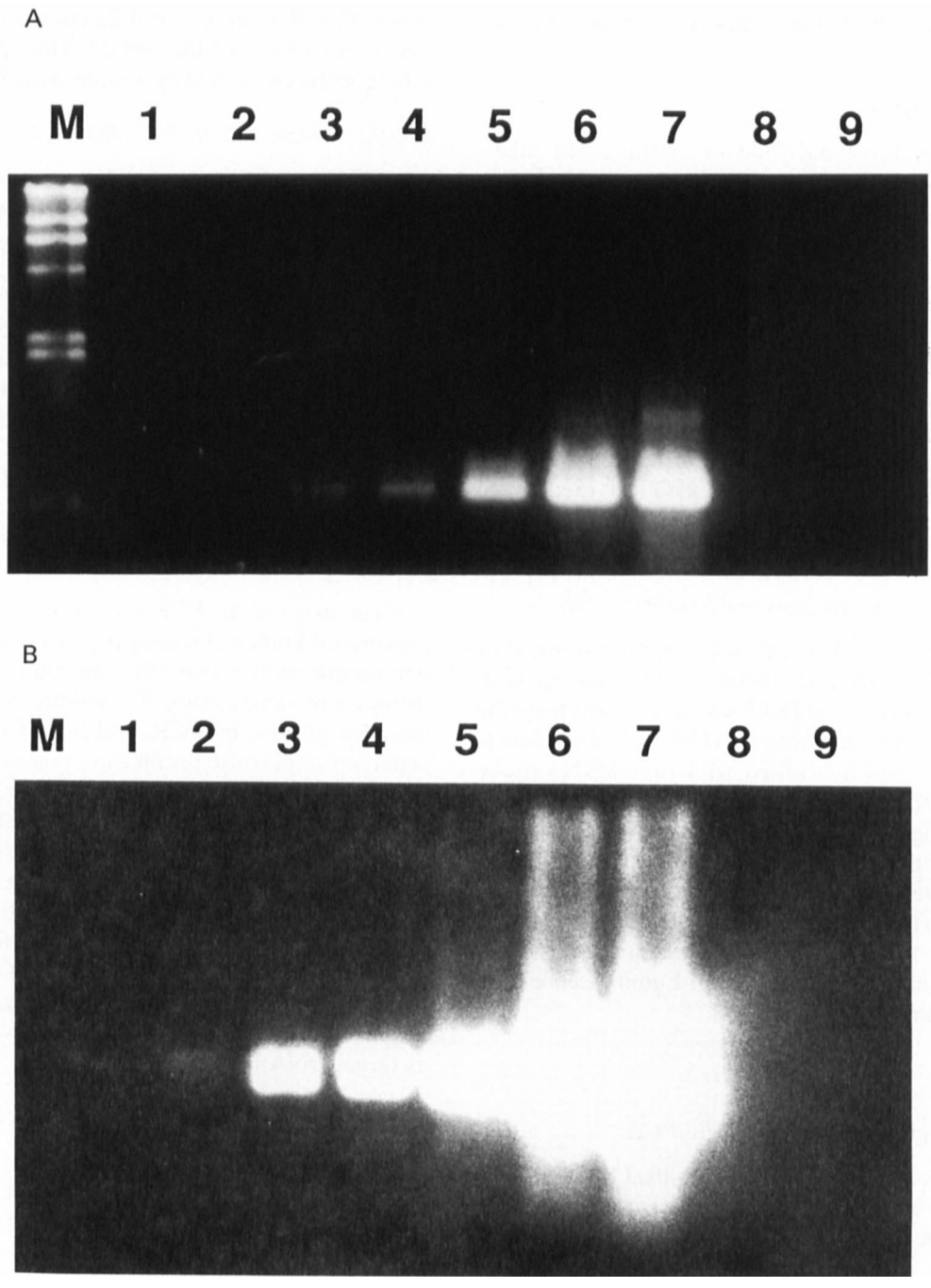

Fig. 2. Sensitivity of the PCR with primer pair B2F and B4R. Agarose 1·2% gel electrophoresis of PCR products amplified from different amounts of *C. albicans*, *E. coli* and human genomic DNA template was done. **A**, stained by ethidium bromide and visualised by UV irradiation; **B**, detection by chemiluminescence; detected by Polaroid 612, ISO 20000 film. Lanes: **M**, *Hin*dIII-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.

same length of DNA products; 10 ng of template DNA was used per reaction and Southern blot analysis was done for confirmation of the results.

To determine the lower limit of detection by PCR with the primer pair B2F and B4R, assays were performed with the genomic DNA of *C. albicans* TIMM2726 serially diluted to give a concentration range of 100 fg–10 ng. Fungal DNA solutions, which were extracted as described by Buchman *et al.*[11] from 100 μl of normal human blood containing EDTA plus 10, 10^1, 10^2, 10^3 or 10^4 cells of *C. albicans* TIMM1768, were also tested by PCR and Southern blot analysis.

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 10^6 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.*[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-μl volumes of the samples were stored at −80°C until needed. DNA extraction was performed as described by Buchman *et al.*[11]

Serodiagnostic tests

Cand-Tec[4] (Ramco, Houston); Pastorex *Aspergillus*[5] (Diagnostics Pastorex, Paris); Serodirect Eiken *Cryptococcus*[5] (Eiken Kagaku Corp., 1-33-8 Hongo, Bunkyo-ku, Tokyo 113 Japan) and G test[6] (Seikagaku Corp., 2-1-5 Hashimoto-cho, Nihonbashi, Chuo-ku, Tokyo 103 Japan) were used as shown in table III.

Agarose gel electrophoresis and Southern analysis

PCR product (5–10 μl) 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+; Amersham), and then hybridised with the 18SIN3 probe labelled with a chemiluminescence detection system (ECL 3′-oligolabelling and detection system; Amersham). 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 *Muc. circinelloides*, *Muc. racemosus*, *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 *Muc. circinelloides*, *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).

Table II. Murine model of *C. albicans* infections

Days after infection	Mouse no.	PCR	Culture	Kidney abscess
0*	1	−	−	−
	2	−	−	−
1	3	+	+	−
	4	+	−	−
2	5	−	−	+
	6	+	−	+
3	7	+	−	+
	8	−	−	+
4	9	+	−	+
	10	+	−	+

*Not infected.

Sensitivity of detection of C. albicans by PCR

PCR with the primer pair B2F and B4R was able to detect 1 pg of *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.[24] PCR with the template extracted from blood samples containing *C. albicans* cells detected 10^2 *C. albicans* cells by ethidium bromide staining and 10 cells by Southern analysis (data not shown). There was no amplified product from blood without *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 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: *C. albicans* from blood; *H. anomala* from cerebrospinal fluid and *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 *Cr. neoformans*. Blood samples from patients 5, 6 and 7 (immunocompromised hosts who were resistant to anti-bacterial 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

Table III. Detection of pathogenic fungi in clinical samples by PCR with the primer pair B2F and B4R

Patient no.	Specimen used for PCR	PCR results	Culture	Diagnostic kit (result)	Clinical diagnosis
1	Blood	+	*C. albicans* Blood(+)	Cand-Tec(−)*	Diabetes mellitus
2	CSF	+	*H. anomala* Blood(+), CSF(+)	Not tested	Meningitis
3	Sputum	+	*A. fumigatus* Sputum(+)	Pastorex *Aspergillus*(−)*	Aspergilloma
4	Blood	+	*Cr. neoformans* Blood(−), CSF(+)	Serodirect Eiken *Cryptococcus* Serum(+), CSF(+) G-test(+)*	Cryptococcosis (meningitis, pneumonia)
5	Blood	+	−	Cand-Tec(−)*, G-test(−)*	Malignant lymphoma
6	Blood	+	−	Cand-Tec(−)*, G-test(−)*	Acute lymphocytic leukaemia
7	Blood	−	−	Cand-tec(−)*	Chronic renal failure

CSF, cerebrospinal fluids.
*Serum was used as a sample.

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,[11–15,23] when purified DNA or cultured cells of *C. albicans* were used as the templates. There are only three reports[11,12,15] referring 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 $> 10^2$ 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.[24,30,31] 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*[22] 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)[32] was also detected by this PCR system. Some reports of detection of *Pn. carinii* with PCR have been published.[16–20] Lipschik *et al.*[16] 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.[11–15,17,20] Diagnostic kits also reveal less sensitivity on tested samples than by our technique.

New technology, such as enzymic detection of PCR products[33] 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.

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